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Award Number: DAMD17-01-1-0555

TITLE: Novel Artificial Natural Products Against Breast Cancer Through Combinatorial Biosynthesis

PRINCIPAL INVESTIGATOR: Jurgen Rohr, Ph.D.

CONTRACTING ORGANIZATION: Medical University of South Carolina
Charleston, South Carolina 29425

REPORT DATE: July 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2002	Final (1 Jul 01 - 30 Jun 02)

4. TITLE AND SUBTITLE
Novel Artificial Natural Products Against Breast Cancer Through Combinatorial Biosynthesis

5. FUNDING NUMBERS
DAMD17-01-1-0555

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Medical University of South Carolina
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
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12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The technique of combinatorial biosynthesis was applied, to generate derivatives (analogs) of various anticancer drugs, including aureolic acids (mithramycin), angucyclins (landomycins, urdamycins, oviedomycin), anthracycline-like compounds (elloramycins), and indolcarbazoles (rebeccamycin). In addition, a novel derivative of mitomycin was synthesized.

The hypothesis could be proven that novel mithramycin analogs with modifications in the sugar moieties and the pentyl side chain could be generated through inactivation of methyltransferase and ketoreductase encoding genes. The recombination of landomycin glycosyltransferase encoding genes with genes of the urdamyciun pathway led to novel urdamycin derivatives, and methyltransferases and the glycosyl transferase of the elloramycin gene cluster were investigated, yielding various new elloramycin analogs. In addition, the gene cluster of the indolcarbazole drug rebeccamycin was unrevealed and investigated, and the novel angucyclinone drug oviedomycin has been discovered through heterologous expression of its gene cluster. Many of the new drugs show antiproliferative activities and are potential drugs against breast cancer.

14. SUBJECT TERMS
combinatorial biosynthesis, glycosyltransferase, methyltransferase ketoreductase, gene cluster, halogenase, tailoring enzymes, breast cancer

15. NUMBER OF PAGES
106

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Combinatorial biosynthesis is the systematic modification and interchange of genes involved in the biosynthesis of natural products with the consequential production of 'unnatural' or 'hybrid' natural products. This concept has progressed dramatically since it was introduced in the late 1980ties. Having started with polyketides, this research has been extended to non-ribosomal peptides, compounds with mixed polyketide/non-ribosomal peptide structural elements and is beginning to include many other kinds of natural products. For several reasons, polyketides remain the central group of natural products in this research area, since this class of natural products form one of the largest and most diverse group of secondary metabolites, which are produced by a myriad of different organisms including various microorganisms, fungi, plants, algae, dinoflagellates, etc. They have an unusually high quote of biologically active compounds among their lines, such as antibiotics, antifungals, anticancer drugs, immunosuppressants, cholesterol lowering agents, analgesics, antiparasitics, neurotoxins, and virulence determinants. However, most approaches in context with combinatorial biosynthesis focus on antibiotics and on anticancer drugs. Typically, the general assembly process, i.e. the early biosynthetic steps are studied, and only a few studies aim on late-acting steps, although those are often responsible for the introduction of biological activity.

Typical late steps in biosyntheses of natural products are oxygenation, reduction, glycosyl- and methyltransfer, and halogenation reactions. These steps provide or change functionality, solubility, and general shape of the affected molecules.

The goal of our research was to apply the concept of 'combinatorial biosynthesis' on anticancer drugs with activity against breast cancer cells. The studies were supposed to focus on late biosynthetic steps, and it was particularly envisaged to generate novel analogs of existing antitumor drugs through modification of oxygenases, ketoreductases, glycosyl transferases, methyltransferases, and halogenases.

Most of our work was performed on aromatic polyketides, since this group provides a large number of currently used natural antitumor drugs, but also other types of natural anticancer drugs were included in our study.

Body

A) Research

1) Aureolic Acid Drugs

The studies focused on the generation of novel mithramycin analogs, and were recently expanded on chromomycin. The studies aimed on the modification of the sugar moieties and the pentyl side chain of mithramycin.

Mithramycin is a polyketide aureolic acid-type antimicrobial and antitumor agent produced by *Streptomyces argillaceus*. Modifying late biosynthetic steps, i.e. post-polyketide synthase (PKS) tailoring enzymes involved in the production of mithramycin is an effective way of gaining further information regarding the late steps of its biosynthetic pathway and to produce new 'unnatural' natural products of the aureolic acid-type class.

The role of two such post-PKS tailoring enzymes, encoded by *mtmC* and *mtmTIII*, were investigated, and four novel aureolic acid class drugs, two premithramycin-type molecules and two mithramycin derivatives, were isolated from mutant strains constructed by insertional gene inactivation of either of these two genes. From data bank comparisons, the corresponding proteins MtmC and MtmTIII were believed to act as a C-methyltransferase involved in the production of the D-mycarose (sugar E) of mithramycin and as a ketoreductase seemingly involved in the biosynthesis of the mithramycin aglycon, respectively. However, gene inactivation and analysis of the accumulated products revealed that both genes encode enzymes participating in the biosynthesis of the D-mycarose building block. Furthermore, the inactivation of MtmC seems to affect the ketoreductase responsible for 4-ketoreduction of sugar C, a D-olivose. Instead of obtaining premithramycin and mithramycin derivatives with a modified

E-sugar upon inactivation of *mtmC*, compounds were obtained that completely lack the E-sugar moiety and that possess an unexpected 4-ketosugar moiety instead of the D-olivose at the beginning of the lower deoxysaccharide chain. The inactivation of *mtmTIII* led to the accumulation of 4E-ketomithramycin, showing that this ketoreductase is responsible for the 4-ketoreduction of the D-mycarose moiety. The new compounds of the mutant strains, 4A-ketopremithramycin A2, 4A-keto-9-demethylpremithramycin A2, 4C-keto-demycarosylmithramycin, and 4E-ketomithramycin, indicate surprising substrate flexibility of post-PKS enzymes of the mithramycin biosynthetic pathway. Although the glycosyltransferase responsible for the attachment of D-mycarose cannot transfer the unmethylated sugar to the existing lower disaccharide chain, it can transfer the 4-ketoform of sugar E. In addition, the glycosyltransferase MtmGIV, which is responsible for the linkage of sugar C, is also able to transfer an activated 4-ketosugar. The oxygenase MtmOIV, normally responsible for the oxidative cleavage of the tetracyclic premithramycin B into the tricyclic immediate precursor of mithramycin, can act on a substrate analog with a modified or even incomplete trisaccharide chain. The same is true for glycosyltransferases MtmGI and MtmGII, both of which partake in the formation and attachment of the A-B disaccharide in mithramycin.[1]

Heterologous expression of the *urdGT2* gene from the urdamycin producer *Streptomyces fradiae* Tü2717, which encodes a *C*-glycosyltransferase, into mutants of the mithramycin producer *S. argillaceus*, in which the natural glycosyltransferase encoding genes were inactivated, yielded four novel *C*-glycosylated premithramycin-type molecules. The structure elucidation revealed these to be 9-*C*-olivosyl-premithramycinone, 9-*C*-mycarosyl-premithramycinone, and their respective 4-*O*-demethyl analogs. In another experiment, both, the *urdGT2* gene from *S. fradiae* as well as the *lanGT1* gene from *S. cyanogenus*, which encodes a special olivosyltransferase, were heterologously co-expressed into a *S. argillaceus* mutant. This experiment, in which genes from three different organisms were combined, resulted in the production of 9-C-(olivo-1-4-olivosyl)-premithramycinone. These results prove the unique substrate flexibility of the C-glycosyltransferase UrdGT2, which tolerates not only a variety of sugar-donor substrates, but also various acceptor substrates. The five new hybrid

products also represent the first compounds, in which sugars were attached to a position, which is normally unglycosylated. The successful combination of two glycosyltransferase encoding genes in the latter experiment proves that the design of saccharide side chains by combinatorial biosynthetic methods is possible.[2]

Inactivation of the putative ketoreductase encoding gene *mtmW* revealed two novel compounds, mithramycin SK and demycarosyl-mithramycin SK. Since previous biosynthetic studies on mithramycin revealed that a ketoreduction step is required for the formation of the pentyl side chain, *mtmW* was one of the remaining candidates likely to encode the enzyme responsible for this important reduction step, and derivatives with a second keto function in the side chain were expected. This was indeed the case, but surprisingly, both novel compounds have a shorter side chain, namely a butyl instead of a pentyl side chain. Although the experiments cannot fully explain this shorter side chain, it is likely that the polyketide synthase was indirectly affected by the inactivation experiment of *mtmW*, or a chemical decarbonylation reaction is responsible for the excision of one carbon and one oxygen atom.

Initial antitumor assays (MTT assays) showed that all newly generated mithramycin drugs are active, usually less active than mithramycin itself, with the exception of mithramycin SK, which has an improved therapeutic index. We are currently in the process to patent mithramycin SK, and more assays (DNA binding assays) with the novel mithramycin analogs are in progress. All premithramycins were inactive so far.

2) Angucyclines

The studies focused initially on the generation of urdamycin/landomycin hybrids, particularly on the modification of sugar residues through recombination of glycosyltransferase encoding genes.

Glycosyltransferases are important biosynthetic enzymes that link sugar moieties to aglycones, which often derive from polyketides. Biological activity is frequently generated along with this process. Here we report the use of glycosyltransferase genes isolated from the landomycin biosynthetic gene cluster to create hybrid landomycin/urdamycin oligosaccharide antibiotics.

Production of several novel urdamycin derivatives by a mutant of *S. fradiae* Tü2717 has been achieved in a combinatorial biosynthetic approach using glycosyltransferase genes from the landomycin producer *S. cyanogenus* S136. For the generation of gene cassettes useful for combinatorial biosynthesis experiments new vectors named pMUNI, pMUNII and pMUNIII were constructed. These vectors facilitate the construction of gene combinations taking advantage of the compatible *Mun*I and *Eco*RI restriction sites.

The high-yielding production of novel oligosaccharide antibiotics using glycosyltransferase gene cassettes generated in a very convenient way proves that glycosyltransferases can be flexible towards the alcohol substrate. In addition, our results indicate that LanGT1 from *S. cyanogenus* S136 is a D-olivosyltransferase, whereas LanGT4 is an L-rhodinosyltransferase. These results were important, since it was also planned to use *lanGT* genes for modifying the mithramycin pathway (see above).[3]

The second project in context with angucycline drugs focused on the discovery of new genes, which can be used for combinatorial biosynthetic studies. Genes that are unexpressed under normal conditions (sometimes called 'silent genes') are discussed in the context of drug discovery strategies. The search for such genes in bacteria of the genus *Streptomyces* seems profitable, since they not only produce the most biologically active natural products, but are also known for their large genome, which often contains far more biosynthetic genes than necessary to encode the biosynthesis of the compounds normally produced by a certain strain.

Our investigations on the discovery of novel natural metabolites using type II polyketide synthase gene probes (*act*I/III) yielded an unusual angucyclinone, oviedomycin, when applied to the oleandomycin producer *Streptomyces antibioticus* ATCC11891. The novel natural product was produced using *S. albus* RM as a host strain, into which a cosmid containing the oviedomycin gene cluster was transformed. Its structure was elucidated by NMR spectroscopy and mass spectrometry. We are currently working on the elucidation of the gene cluster of this novel natural product, and hope to find useful genes for combinatorial biosynthesis studies.[4]

3) Anthracycline-Like Compounds

These studies focused on the exploration of the unusual glycosyltransferase ElmGT, which seemed to be capable of transferring seemingly any kind of activated sugar. The products are glycosylated tetracenomycins (called elloramycins), since the alcohol acceptor substrate of the enzyme is always 8-*O*-demethyltetracenomycin C (8-DMTC). This research serves the general aspect to understand the possibilities and limits of combinatorial biosynthesis on sugar subunits. The ElmGT glycosyltrasnferase from *S. olivaceus* Tü2353 can transfer different sugars to the aglycon 8-DMTC. In addition to its natural sugar donor substrate L-rhamnose, ElmGT can transfer several L- and D-sugars ans also a dioloivosyl disaccharide to 8-DMTC. Thus, ElmGT is an example of a unique sugar substrate flexible glycosyltransferase and can represent an important tool for combinatorial biosynthesis. Using degenerated oligoprimers derived from conserved amino acid sequences in glycosyltransferases, the gene encoding this sugar donor substrate flexible glycosyltransferase has been identified, and was heterologously expressed to transfer various monosaccharides (both D- and L-sugars) to 8-DMTC. [5]

Our newest explorations of glycosyltransferase ElmGT were extended towards various previously unexplored sugar co-substrates. The studies revealed that ElmGT, which normally transfers L-rhamnose to 8-demethyltetracenomycin C as a crucial biosynthetic step in the elloramycin biosynthesis, is also able to process an activated non-deoxygenated sugar, NDP-D-glucose as well as NDP-L-digitoxose, which is the first example of an NDP-L-sugar co-substrate of ElmGT possessing an axial 3-OH group. The structures of the resulting novel elloramycin analogs of these experiments, 8-demethyl-8-L-digitoxosyl-tetracenomycin C and 8-demethyl-8-D-glucosyl-tetracenomycin C, were elucidated mainly by ^{1}H and ^{13}C NMR spectroscopy and by mass spectrometry.[6]

4) Other Anticancer Drugs

The research described in this paragraph reflects our recent attempts to expand combinatorial biosynthesis from polyketides to other classes of natural products, namely on amino acid

derived drugs. The first project aimed on the finding and elucidation of the gene cluster of rebeccamycin, a representative of the indolocarbazole antitumor drugs, the second project dealt with the synthesis of a novel mitomycin derivative.

Rebeccamycin is a halogenated natural product of the indolocarbazole family, produced by *Saccharothrix aerocolonigenes* ATCC39243. Several rebeccamycin analogues, which are DNA-damaging agents targeting DNA topoisomerase I or II, have already entered clinical trials as anticancer drugs. Using as a probe an internal fragment of *ngt*, a gene from *Saccharothrix aerocolonigenes* encoding an indolocarbazole *N*-glycosyltransferase, a DNA region was isolated that directed the biosynthesis of rebeccamycin, with a substantial increase in yield, when introduced in *Streptomyces albus*. Sequence analysis of 25.6 kb revealed candidate genes for indolocarbazole core formation, halogenation, glycosylation and sugar methylation, as well as a regulatory gene and two resistance/secretion genes. Heterologous expression of subsets of these genes resulted in production of deschloro-rebeccamycin, 4'-demethyl-deschloro-rebeccamycin, and deschloro-rebeccamycin aglycone. The cloned genes should help to elucidate the molecular basis for indolocarbazole biosynthesis and set the stage for generation of novel indolocarbazole analogues by genetic engineering.[7]

A novel conjugate of mitomycin C (MMC) and triamcinolone acetonide (TA) was synthesized using glutaric acid as a linker molecule. To determine the rate of hydrolysis, the conjugate was dissolved in aqueous solution and the rate of appearance of free MMC and TA determined by HPLC analysis. Antiproliferative activity of the MMC-TA conjugate and parent compounds were assessed using an NIH 3T3 fibroblast cell line. Cell growth was quantified using the MTT assay. Kinetic analysis of the hydrolysis rate demonstrated that the conjugate had a half-life of 23.6 hours in aqueous solutions. The antiproliferative activity of the MMC-TA conjugate and MMC were both concentration dependent, with similar IC_{50s} of 2.4 μM and 1.7 μM, respectively. However, individual responses at concentrations above 3 μM showed that the conjugate was less active than MMC alone. TA alone showed only limited inhibition of cell growth. Our data provide evidence that the MMC-TA conjugate could be used as a slow-release drug delivery

system. This could in turn be used to modulate a post-treatment wound healing process or to treat various proliferative diseases.[8]

B) Involved Personnel

The following person's salaries were paid or partly paid with the DOD Army grant:

1) Lily L. Remsing (graduate student)

2) Dr. Igor Butovich

3) Carsten Fischer (graduate student)

Key Research Accomplishments

Focusing on glycosyltransfer, methyltransfer, and oxidoreductases, 23 new compounds could be generated, most of them aureolic acid (mithramycin, premithramycin) derivatives. The generation of other compounds, such as angucycline drugs, was intended to flank the ultimate goal, creating new aureolic acid drugs. For instance, the work in which the glycosyltransferase encoding genes of the landomycin pathway were identified,[3] led ultimately to the generation of premithramycin-type drugs.[2] The evaluation of these new compounds, such as proliferation inhibition studies, toxicity assays and DNA interaction studies, is in progress. Various new genes were discovered, which ultimately may be useful for further gene recombination attempts.

Reportable Outcomes

The studies so far yielded eight publications (see references, and appendix) in top-ranking scientific journals, in which the DOD funding was acknowledged, one patent (in preparation), and one NIH grant (RO1CA91901).

Conclusions

The studies supported by this DOD grant showed that it is profitable for combinatorial biosynthetic approaches to include tailoring enzymes catalyzing late biosynthetic steps of diverse natural product biosyntheses.

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8) T. Macky, C. Oelkers, U. Rix, M. L. Heredia, E. Künzel, M. Wimverley, B. Rohrer, C. Crosson, J. Rohr*: Synthesis, pharmacokinetics and rat retinal toxicity of a novel mitomycin C-triamcinolone acetonide conjugate. *J. Med. Chem.* **2002**, *45*, 1122-1127.

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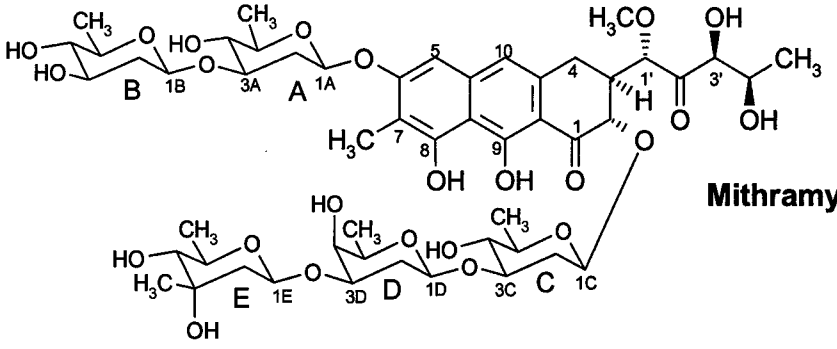
* corresponding author(s)

Appendices

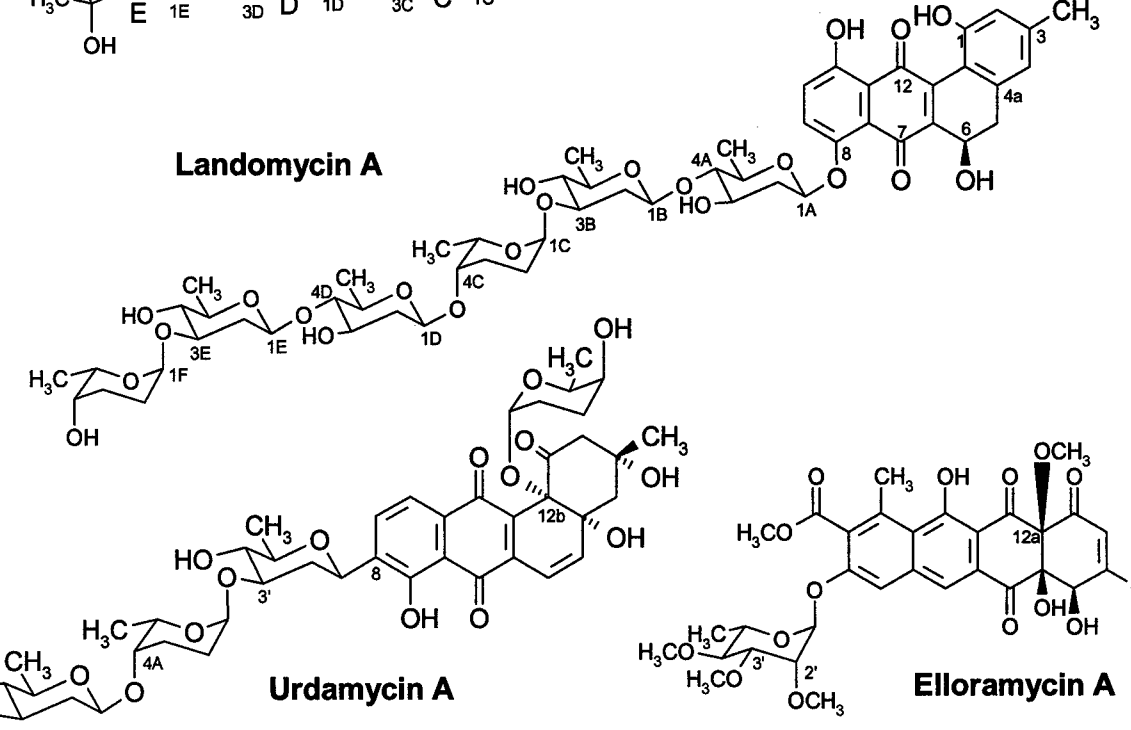
a) Chemical structures of parent drugs (mithramycin, landomycin A, urdamycin A, elloramycin A, rebeccamycin, mitomycin C, scheme 1, page 14) and novel 'unnatural' analogs/derivatives generated by combinatorial biosynthesis (schemes 2-4, pages 15-17).

b) Seven reprints of publications, one manuscript (accepted by *J. Nat. Prod.*), following page 17, not numbered.

Scheme 1: Parent Natural Antitumor Drugs



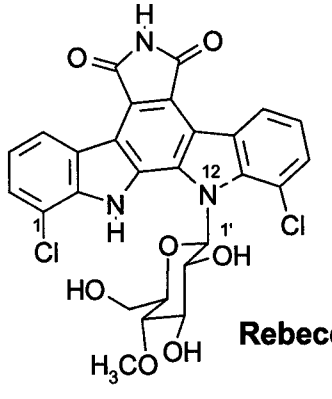
Mithramycin



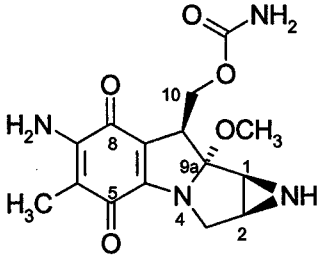
Landomycin A

Urdamycin A

Elloramycin A

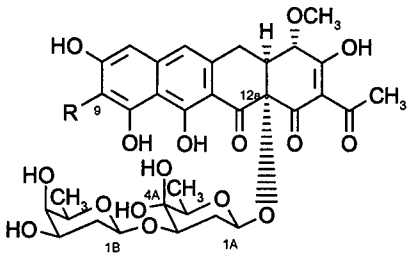


Rebeccamycin

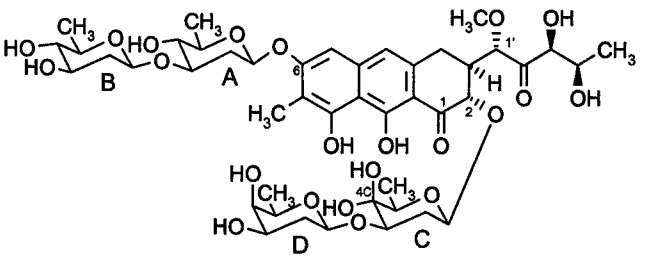


Mitomycin C

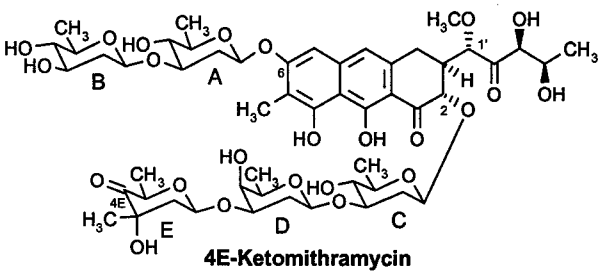
Scheme 2: Aureolic Acid Derivatives/Analogs



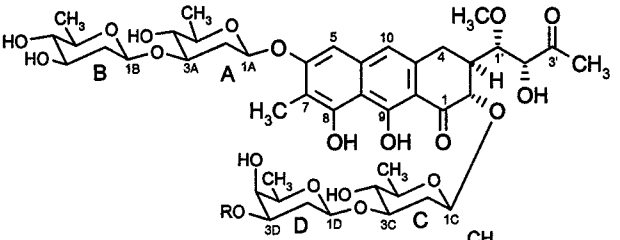
4A-Ketopremithramycin A2 R = CH_3
4A-Keto-9-demethylpremithramycin A2 R = H



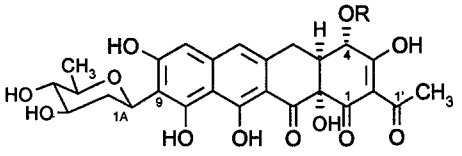
4C-Keto-demycarosylmithramycin



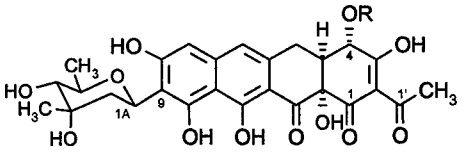
4E-Ketomithramycin



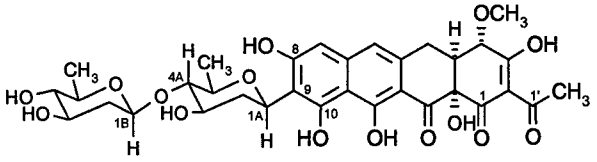
Mithramycin SK: R =
Demycarosyl-mithramycin SK: R = H



9-C-olivosylpremithramycinone: R = H
9-C-olivosyl-4-O-demethyl-premithramycinone: R = CH_3

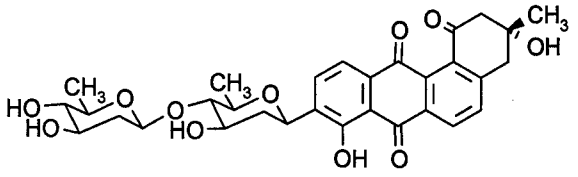


9-C-mycarosylpremithramycinone: R = H
9-C-mycarosyl-4-O-demethyl-premithramycinone: R = CH_3

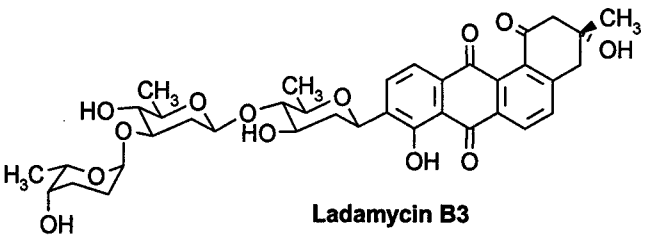


9-C-(olivo-1-4-olivosyl)premithra-mycin one

Scheme 3: Angucyclin-Derivatives



Ladamycin B2



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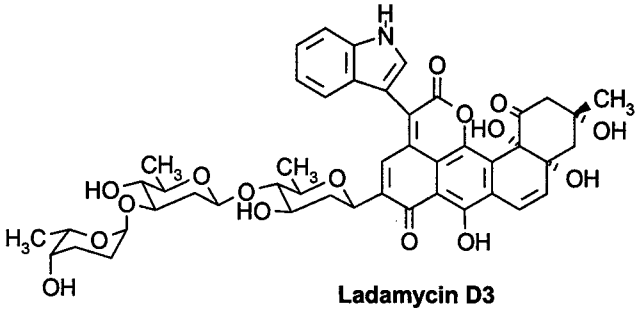
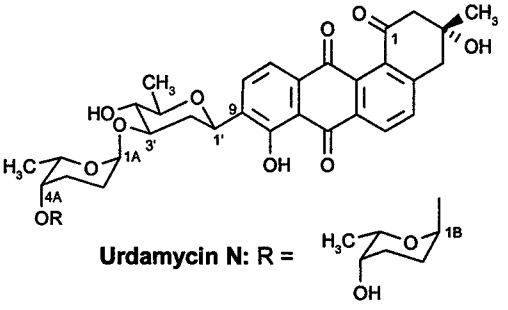
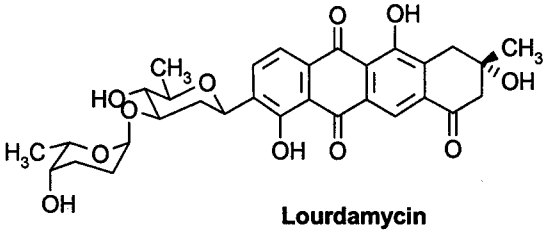


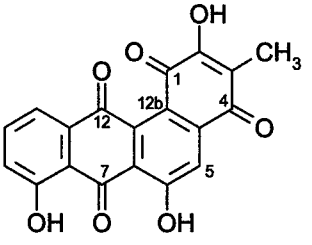
Ladamycin D3



Urdamycin N: R =

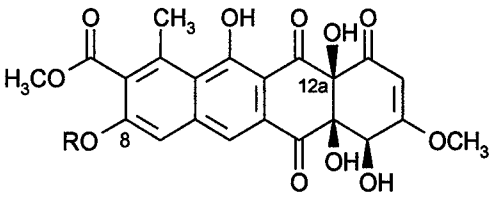


Lourdamycin

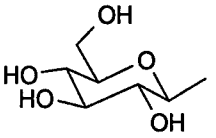


Oviedomycin

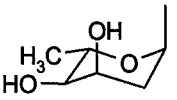
Scheme 4: Elloramycin-Derivatives and Amino Acid Derived Drug-Derivatives

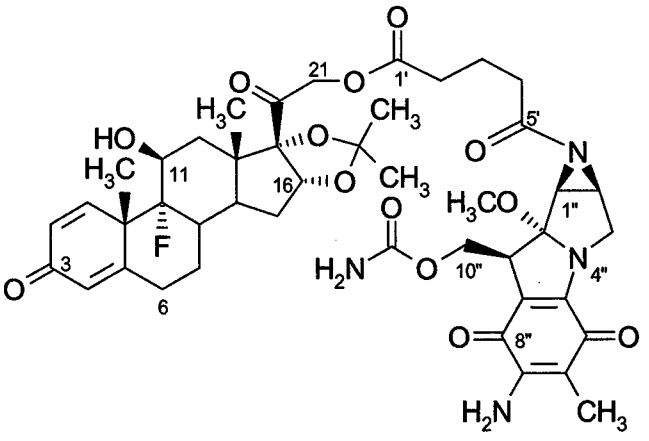


8-Demethyl-8-D-glucosyltetracenomycin C: R =

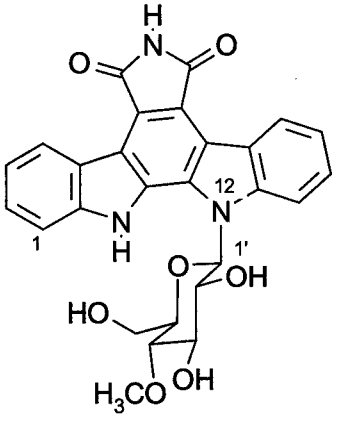


8-Demethyl-8-L-digitoxosyltetracenomycin C: R =

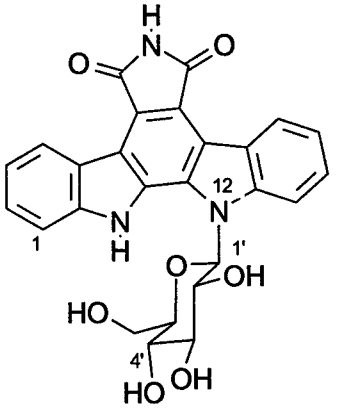




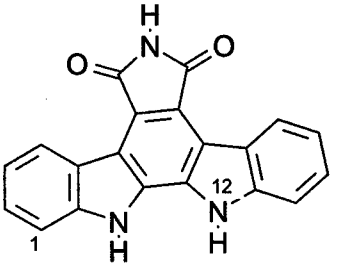
Mitomycin-Triamcinolone Conjugate



Deschlororebeccamycin



4'-Demethyl-deschlororebeccamycin



Deschlororebeccamycin-aglycone

Ketopremithramycins and Ketomithramycins, Four New Aureolic Acid-Type Compounds Obtained upon Inactivation of Two Genes Involved in the Biosynthesis of the Deoxysugar Moieties of the Antitumor Drug Mithramycin by *Streptomyces Argillaceus*, Reveal Novel Insights into Post-PKS Tailoring Steps of the Mithramycin Biosynthetic Pathway

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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY®

Reprinted from
Volume 124, Number 8, Pages 1606–1614

J|A|C|S
ARTICLES
Published on Web 01/30/2002

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Received February 26, 2001

Abstract: Mithramycin is an aureolic acid-type antimicrobial and antitumor agent produced by *Streptomyces argillaceus*. Modifying post-polyketide synthase (PKS) tailoring enzymes involved in the production of mithramycin is an effective way of gaining further information regarding the late steps of its biosynthetic pathway. In addition, new "unnatural" natural products of the aureolic acid-type class are likely to be produced. The role of two such post-PKS tailoring enzymes, encoded by *mtmC* and *mtmTIII*, was investigated, and four novel aureolic acid class drugs, two premithramycin-type molecules and two mithramycin derivatives, were isolated from mutant strains constructed by insertional gene inactivation of either of these two genes. From data bank comparisons, the corresponding proteins MtmC and MtmTIII were believed to act as a C-methyltransferase involved in the production of the D-mycarose (sugar E) of mithramycin and as a ketoreductase seemingly involved in the biosynthesis of the mithramycin aglycon, respectively. However, gene inactivation and analysis of the accumulated products revealed that both genes encode enzymes participating in the biosynthesis of the D-mycarose building block. Furthermore, the inactivation of MtmC seems to affect the ketoreductase responsible for 4-ketoreduction of sugar C, a D-olivose. Instead of obtaining premithramycin and mithramycin derivatives with a modified E-sugar upon inactivation of *mtmC*, compounds were obtained that completely lack the E-sugar moiety and that possess an unexpected 4-ketosugar moiety instead of the D-olivose at the beginning of the lower deoxysaccharide chain. The inactivation of *mtmTIII* led to the accumulation of 4E-ketomithramycin, showing that this ketoreductase is responsible for the 4-ketoreduction of the D-mycarose moiety. The new compounds of the mutant strains, 4A-ketopremithramycin A2, 4A-keto-9-demethylpremithramycin A2, 4C-keto-demycarosylmithramycin, and 4E-ketomithramycin, indicate surprising substrate flexibility of post-PKS enzymes of the mithramycin biosynthetic pathway. Although the glycosyltransferase responsible for the attachment of D-mycarose cannot transfer the unmethylated sugar to the existing lower disaccharide chain, it can transfer the 4-ketoform of sugar E. In addition, the glycosyltransferase MtmGIV, which is responsible for the linkage of sugar C, is also able to transfer an activated 4-ketosugar. The oxygenase MtmOIV, normally responsible for the oxidative cleavage of the tetracyclic premithramycin B into the tricyclic immediate precursor of mithramycin, can act on a substrate analogue with a modified or even incomplete trisaccharide chain. The same is true for glycosyltransferases MtmGI and MtmGII, both of which partake in the formation and attachment of the A-B disaccharide in mithramycin.

Introduction

Mithramycin (**10**, also known as plicamycin), a natural product of *Streptomyces argillaceus* (ATCC 12956) and other *Streptomyces* spp., along with chromomycin A_3, olivomycin A, chromocyclomycin, and UCH9, belongs to the discrete group of aureolic acid anticancer antibiotics.[1−19] The aureolic acid antibiotics are effective against a variety of experimental and human tumors.[1−4,20−25] Mithramycin has been used clinically

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10.1021/ja0105156 CCC: $22.00

to treat testicular carcinoma,[1,3,4,19] Paget's bone disease, and other bone growth disorders.[26-29] In particular, its hypocalcemic effect has been used to manage hypercalcemia in patients with malignancy-associated bone lesions.[30] Unfortunately, **10** exhibits gastrointestinal, hepatic, renal, and bone marrow toxicity resulting in nausea, vomiting, and bleeding.[1,3,29,31] Therefore, its widespread clinical use is limited, making the identification of improved therapeutic mithramycin analogues an important endeavor.

The biosynthetic pathway of **10** has been studied in our laboratories over the past years.[4,32-44] On the basis of the knowledge gained thus far, alteration of the biosynthetic pathway provides a mechanism through which modified **10** analogues can be produced "naturally". We use this approach to target mainly post-polyketide synthase (post-PKS) modifying enzymes, which allow modifications to be made in regions of the molecule identified as crucial for the activity of **10**. The mechanism of action of **10** and in particular its DNA binding have been extensively studied. Mithramycin inhibits replication and transcription processes via cross-linking of DNA strands, thereby blocking their template activity for DNA- and RNA-dependent polymerases. It was shown that the deoxysaccharide moieties and the highly functionalized pentyl side chain play major roles[2,7-9,19,23,24,28,45] in the DNA binding of **10**. In particular, the trisaccharide residue is positioned inside of the DNA minor groove with the D-mycarose moiety sitting in its floor and interacting through H-bonds to both DNA strands. This suggests that changes in the trisaccharide segment may affect binding affinity and sequence selectivity of new **10** analogues. Consequently, the aim of our studies described in this article was to modify the D-mycarose moiety found at the end of the trisaccharide chain.

Past biosynthetic studies revealed to a great extent the sequence of events and role of important post-PKS oxygenases and group transferases in the mithramycin biosynthetic pathway. Inactivation of the four glycosyltransferases (GT) and elucidation of the accumulated products of the resulting mutant strains showed that the GTs MtmGI and MtmGII are involved in the assembly and attachment of the diolivoside fragment A-B,[36] while MtmGIII and MtmGIV participate in the assembly of the trisaccharide chain, linking sugar D (a D-oliose) and sugar C (a D-olivose), respectively (see Figures 1 and 2).[41] The studies also revealed that the disaccharide A-B can be attached only after the trisaccharide chain has been completed, suggesting the biosynthetic sequence **1** → **3** → **6** → **8** → **9** (Figures 1 and 2). Currently, no GT has been identified for the linkage of D-mycarose, the last sugar of the trisaccharide chain, and so there seemed to be no way to selectively modify the trisaccharide by inhibiting its attachment or to investigate the substrate specificity of this GT.

However, three methyltransferases (MT), MtmMI, MtmMII, and MtmC, are present in the *mtm* gene cluster. Recent investigations on the role of MtmMI and MtmMII identified them as being involved in the biosynthesis of the aglycon. MtmMI is the 4-O-methyltransferase responsible for the conversion of 4-demethylpremithramycinone into premithramycinone (**1**, Figure 1) prior to the sugar attachments. MtmMII is the C-methyltransferase, which introduces the aromatic methyl group predominantly after completion of the trisaccharide (product **8**), but occasionally also after attachment of the first or second sugar of the trisaccharide chain.[42] The role for the third potential MT in the *mtm* gene cluster (MtmC) seemed to be the addition of the one remaining methyl group in **10**, specifically that of the mycarose moiety. Thus, it was decided to inactivate MtmC with the hope of revealing a mechanism

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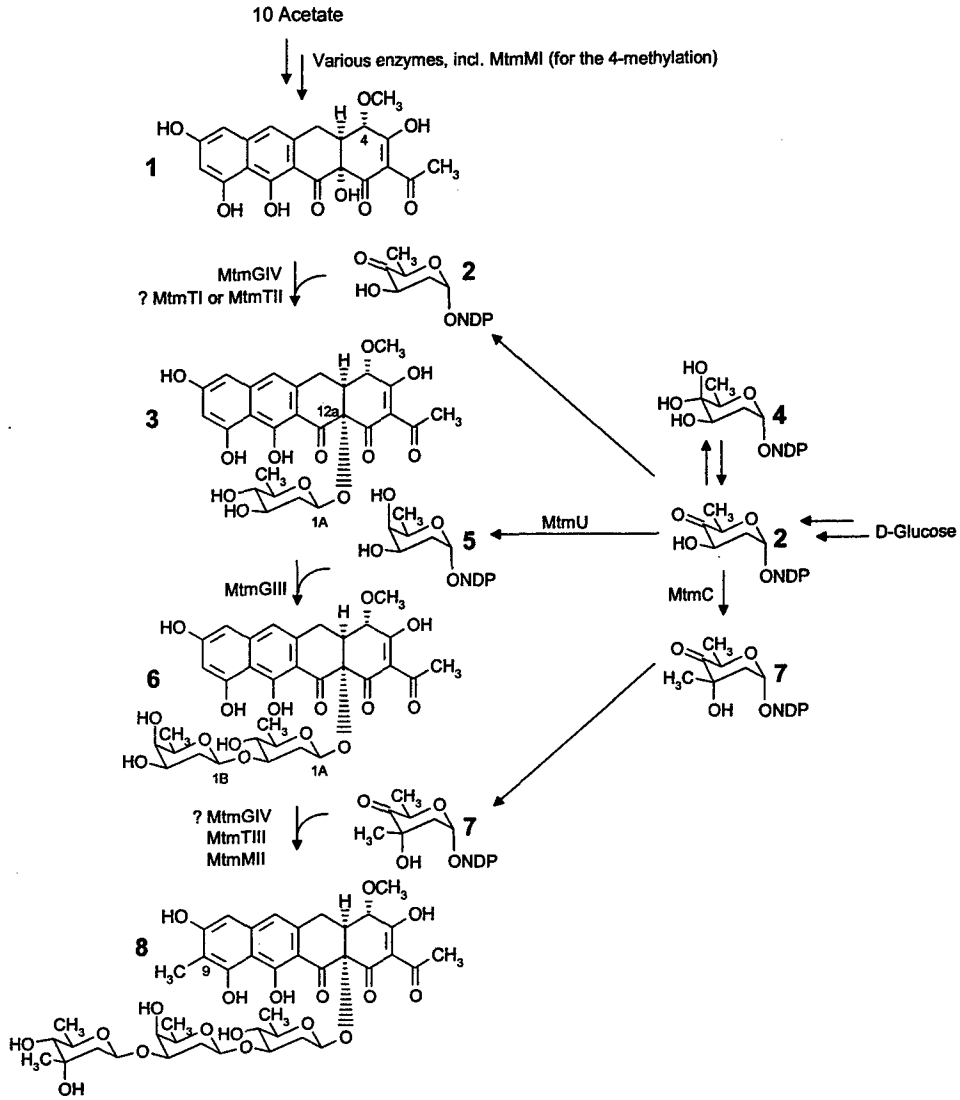

Figure 1. Late biosynthetic steps of the mithramycin (**10**) biosynthetic pathway, including the glycosyltransfer steps, the oxidative cleavage of the fourth ring, and some biosynthetic steps of the deoxysugar units.

through which the third sugar could be modified or eliminated. During these studies, the role of the ketoreductase MtmTIII came into question. It seemed that MtmTIII, whose corresponding gene *mtmTIII* is clustered with the deoxysugar biosynthetic genes and is located immediately upstream of *mtmC* in the same reading direction, may be responsible for the 4-ketoreduction of sugar C (a D-olivose). To investigate this hypothesis, MtmTIII was also inactivated.

Results

The deduced amino acid sequence of MtmC closely resembles various methyltransferases, most of which are involved in deoxysugar biosynthesis. The highest identity was found with SnoG from the nogalamycin pathway in *S. nogalater* (62.2%),[54] DnrX from the daunorubicin pathway in *S. peucetius* (37%),[55] TylCIII from the tylosin pathway in *S. fradiae* (32.4%),[56,57] and EryBIII from the erythromycin pathway in *Sacc. erythraea* (31%).[58] TylCIII and EryBIII have been proposed to be *S*-adenosylmethionine-dependent methyltransferases involved in C-3-methylation during the biosynthesis of L-mycarose. On the basis of these similarities, we predicted that *mtmC* may encode a C-3-methyltransferase involved in the biosynthesis of the D-mycarose moiety of mithramycin. To prove this hypothesis,

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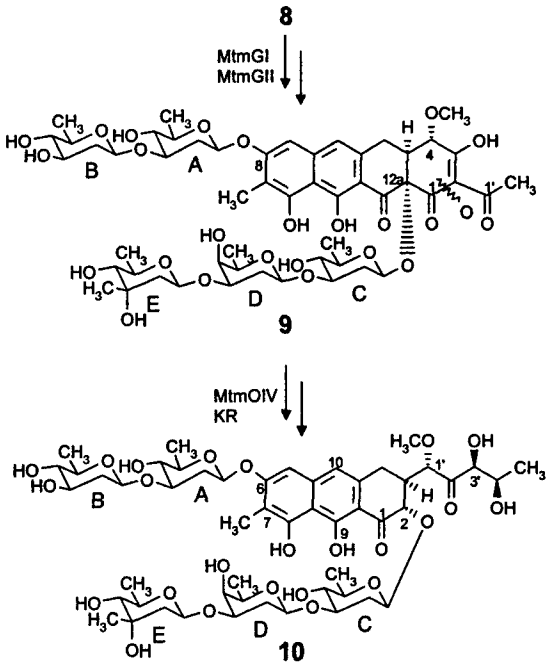


Figure 2. Late biosynthetic steps of the mithramycin (**10**) biosynthetic pathway, including the glycosyltransfer steps, the oxidative cleavage of the fourth ring, and some biosynthetic steps of the deoxysugar units.

mtmC was inactivated by gene replacement.[44] An apramycin resistance cassette was inserted within the gene in both the same and the reverse orientations with respect to the transcriptional direction of *mtmC* (M7C1 and M7C1R). As expected, HPLC analysis of cultures of both mutants showed that they did not produce **10**. Instead, two identical compounds accumulated in both strains, while a third compound was detected only in M7C1.

The compounds were purified by preparative HPLC, and their structures were determined by NMR and mass spectroscopy. The major compound of both mutant strains exhibits all of the typical NMR signals of a tetracyclic premithramycin-type aglycon including the 9-methyl group, but only has two sugar moieties present. Unexpectedly, one of these sugars contains a hydrated keto group in the 4-position. This is strongly supported by the NMR data (Tables 1 and 2), which reveal a quaternary carbon in the ^{13}C NMR spectrum (δ_C 90.6, no signal in the HSQC spectrum) and no proton in the 4-position (^1H NMR). The H,H-COSY, HSQC, and HMBC data (Table 3) allow the unambiguous assignment of all of the carbons and protons of this 4-ketosugar and also, through the observation of the $^3J_{C-H}$ coupling between C-12a and 1-H of the sugar in the HMBC spectrum, indicate its linkage to the 12a-position of the aglycon. The H,H coupling constants, along with the other ^1H and ^{13}C NMR data, prove the other sugar moiety to be an oliose. The presence of only small couplings to the 4B-H indicating the axial position of the 4-OH group is most important. The oliose is linked with its C-1 to the oxygen in the 3-position of the ketosugar, as implied by the $^3J_{C-H}$ couplings between its 1-H and C-3 of the ketosugar as well as between its C-1 and 3-H of the ketosugar moiety visible in the HMBC spectrum (Table 3). The negative FAB mass spectrum shows a molecular ion ($M-H^-$ 703, 100%, HR calculated for $C_{34}H_{39}O_{16}$, 703.2238; found, 703.2206), which corresponds to a mass difference of 16 amu from premithramycin A2 (9-methyl-**6**)[36] and thus supports the structure with the hydrated keto group. Therefore, the main compound is 4A-ketopremithramycin A2 (**11**, Figure 3).

Compared to this compound, the minor compound found in both mutant strains differs only in that it is missing the aromatic 9-methyl group. Instead, 9-H can be observed in the ^1H NMR spectrum at δ 6.45. The chemical shifts and coupling constants of all other ^1H NMR signals are almost identical with the major compound allowing us to suggest 4A-keto-9-demethylpremithramycin A2 (**12**) as the structure for this minor compound.

The third compound, which is only observed as a product of the M7C1 mutant, exhibits the typical UV light (366 nm)-induced yellow fluorescence of the tricyclic mithramycin chromophore. Indeed, the NMR data reveal a compound that possesses the tricyclic mithramycinone skeleton, but only four deoxysugar building blocks. Comparison with the mithramycin NMR data[11,37,39] indicates that the A-B disaccharide attached at C-6 is unchanged, while the lower chain attached at C-2 is identical to the disaccharide containing the hydrated 4-ketosugar found in **11** and **12**. Thus, 4C-keto-demycarosylmithramycin (**13**) was deduced as the structure of this third compound. Long-range couplings observed in the HMBC spectrum (Table 3) as well as the negative FAB mass spectrum (M^- 956, 100%) confirm these conclusions.

Interestingly, all three novel compounds discovered by inactivation of *mtmC* are chemically in a keto/hydrate equilibrium. The keto form of the olivose attached at C-12a and C-2, respectively, is the minor form (10−20%), visible in the ^1H NMR spectrum (shifted 1-, 3-, and 5-positions, data not shown). The keto form is not detectable in the ^{13}C NMR. Changing the solvent from acetone to DMSO increases the amount of the keto form (data not shown), which becomes dominant after being dissolved in DMSO for 72 h.

While the absence of the D-mycarose moiety in all three new compounds is in agreement with the proposed function for MtmC as the C-3-methyltransferase necessary for the formation of the D-mycarose unit (see Discussion), the appearance of a hydrated keto group at the 4-position of the first sugar of the lower saccharide chain (normally a D-olivose, as in **10**) was unexpected and intriguing. This suggested that in both mutants another enzymatic function, a sugar 4-ketoreductase, was also affected. Our initial interpretation was that the inactivation of *mtmC* was causing a polar effect on genes transcribed in the same direction and following *mtmC*.[34,36,38−42] Since a potential ketoreductase gene, *mtmTIII*, is located adjacent to *mtmC* and immediately upstream in the same reading direction, this gene was consequently thought to be most likely affected.

To further clarify this situation, two experiments were carried out: (i) complementation experiments with *mtmC* cloned in a multicopy plasmid using the M7C1 and M7C1R mutants as transformation hosts, and (ii) inactivation of *mtmTIII*. In the first experiment, overexpression of *mtmC* restored the production of mithramycin in both the M7C1R and the M7C1 mutants.[44] The anticipated 4C-ketomithramycin was not produced. Thus, it is unlikely that the presence of the 4-keto group of sugar A in **11** and **12** and sugar C in **13** is caused by a polar effect. For the second experiment, the *mtmTIII*-minus mutant was constructed by inserting an apramycin resistance cassette within the *mtmTIII* gene (Figure 4). This mutant (M7T3) yielded only one compound, a mithramycin derivative, which was subsequently identified as 4E-ketomithramycin (**14**). The negative

Table 1. ^{1}H NMR Data of 4A-Ketopremithramycin A2 (**11**), 4A-Keto-9-demethylpremithramycin A2 (**12**), 4C-Keto-demycarosylmithramycin (**13**), and 4E-Ketomithramycin (**14**) in Acetone-d_6 at 400 MHz (δ in ppm Relative to Internal TMS)[a]

	δ multiplicity (J/Hz)			
	11	**12**	**13**	**14**[c]
2-H			4.70 d (12)	
3-H			2.78 br t (12)	4.80 d (12)
4-H	4.24 s br	4.31 s br	a: 2.92 dd (16)[b]	2.82 br t (12)
			e: 2.60 dd (16,3)	a: 3.00 complex
4-OCH_3	3.6 s	3.61 d (1)		e: 2.68 dd (16,3)
4a-H	3.13 ddd (11,4,3)	3.13 ddd (11,4,3)		
5-H	a: 3.95 (16,4,1)	3.96 d br (16)	6.81 s	6.86 s
	e: 3.05 (16,3)	3.07 obsc		
6-H	6.97 d (1)	7.03 s br		
7-H	6.75 s	6.69 s		
7-CH_3			2.09 s	2.15 s
9-H		6.45 s		
9-CH_3	2.19 s			
10-H			6.85 s	6.84 s
1′			4.88 d (1.5)	4.88 d (2)
1′-OCH_3			3.40 s	3.44 s
2′	2.65 s	2.64 s		
3′			4.24 d (3)[b]	4.30 d (3)[b]
4′			4.23 dq (6.5,3)[b]	4.30 dq (6,3)[b]
5′			1.22 d (6.5)	1.27 d (6)
1A	4.94 dd (10,2)	4.95 dd (10,2)	5.34 dd (10,2)	5.33 dd (10,2)
$2A_a$	1.86 ddd (12,12,10)	1.85 ddd (12,12,10)	1.80 ddd (12,12,10)	1.85 ddd (12,12,10)
$2A_e$	2.45 m br	2.44 ddd (12,5,2,)	2.43 ddd (12,5,2)[b]	2.40 ddd (12,5,2)[b]
3A	3.70 dd (12,5)[b]	3.71 dd (12,5)[b]	3.81[b]	3.70[b]
4A			3.06 dd (9,9)	3.06 dd (9,9)
5A	3.45 q (6)	3.44 q (6)[b]	3.52 dq (9,6)	3.51 dq (9,6)
5A-CH_3	1.25 d (6)	1.25 d (6)	1.26 d (6)	1.30 d (6)
1B	4.68 dd (10,2)	4.69 dd (10,2)	4.74 dd (10,2)	4.72 dd (10,2)
2B	1.70 ddd (12,12,10)	1.73 ddd (12,12,10)	a: 1.55 ddd (12,12,10)	1.58 ddd (12,12,10)
	1.90 ddd (12,5,2)	1.92 ddd (12,5,2)	e: 2.19 ddd (12,5,2)	2.20 ddd (12,5,2)
3B	3.82 ddd (12,5,3)	3.83 m[b]	3.6 ddd (12,9,5)	3.60 ddd (12,9,5)
4B	3.52 s br	3.50 s br	2.99 dd (9,9)	3.01 dd (9,9)
5B	3.70 q (6.5)	3.70 q (6.5)	3.40 dq (9,6)	3.41 dq (9,6)
5B-CH_3	1.25 d (6.5)	1.25 d (6.5)	1.26 d (6)	1.30 d (6)
1C			5.09 dd (10,2)	5.14 dd (10,2)
$2C_a$			1.88 ddd (12,12,10)	1.62 ddd (12,12,10)
$2C_e$			2.42 ddd (12,5,2)[b]	2.55 ddd (12,5,2)
3C			3.84 dd (12,5)	3.70 complex
4C				3.02 dd (9,9)
5C			3.40 q (6)	3.32 dq (9,6)
5C-CH_3			1.26 d (6)	1.30 d (6)
1D			4.74 dd (10,2)	4.7 dd (10,2)
$2D_a$			1.80 ddd (12,12,10)	1.76 ddd (12,12,10)
$2D_e$			1.98 ddd (12,5,2)	1.93 ddd (12,5,2)
3D			3.81 ddd (12,5,3)[b]	3.82 complex
4D			3.56 s br	3.54 s br
5D			3.70 q br (6.5)	3.74 q (6)
5D-CH_3			1.26 d (6.5)	1.24 d (6)

[a] br = broad; obsc = obscured by solvent or water; OH signals not visible due to 5% water in the samples. [b] Complex, partially overlapped by other signal(s). [c] Sugar E signals: 1-E, 5.42 dd (10,2); 2-E_a, 1.98 dd (14,10); 2-E_e, 2.30 dd (14,2); 3-E-CH_3, 1.31 s; 5-E, 4.66 q (6); 5E-CH_3, 1.30 d (6).

APCI-MS reveals an $(M-H)^-$ peak at *m/z* 1081.3 (31%), which is 2 amu smaller than the corresponding peak for mithramycin (**10**) and in agreement with a calculated molecular formula of $C_{52}H_{74}O_{24}$, which lacks two protons as compared to the mithramycin molecular formula. While most of the NMR signals of this novel compound **14** are very similar to those of mithramycin,[11,37,39] significant differences can be seen for the signals of sugar E, which is a 4-keto-D-mycarose in **14** and D-mycarose in **10**. Most obvious, the signal for C-4E (δ 76.1 in **10**) is shifted downfield to δ 206.0 in **14**, typical for a keto group. This keto group signal shows $^3J_{C-H}$ couplings to the 3E- and 5E-methyl group protons (see Table 3), which proves its location at the 4-position. Other significant shift differences for C-1E, C-3E, and C-5E as well as for the protons attached to these carbons were also observed, all in agreement with a 4E-carbonyl group. In contrast to the keto group in the 4C-position mentioned in context with structure **13**, the 4E-keto group in **14** clearly appears as a carbonyl, not as a hydrate (keto:hydrate $\approx$ 95:5), as the ^{13}C NMR spectrum shows no signal in the δ 90 region.

Discussion

The results of the above-described experiments clearly point out that MtmC is the 3-C-methyltransferase necessary for the biosynthesis of the D-mycarose moiety of mithramycin. This is supported by its sequence similarity to TylC3 (TylCIII) and EryBIII, other proteins that act as methyltransferases involved in L-mycarose building block biosyntheses. Particular proof came from the inactivation of the *mtmC* gene leading to the accumulation of compounds lacking the D-mycarose moiety of the trisaccharide chain. It seems that the glycosyltransferase responsible for the attachment of D-mycarose to the preceding

Table 2. ^{13}C NMR Data of 4A-Ketopremithramycin A2 (**11**), 4A-Keto-9-demethylpremithramycin A2 (**12**), 4C-Keto-demycarosylmithramycin (**13**), and 4E-Ketomithramycin (**14**) in Acetone-d_6 at 125.7 MHz (δ in ppm Relative to Internal TMS, Assignments Were Made with the Help of Couplings Determined in the HSQC and HMBC Spectra)[a]

	δ					δ			
	11	**12**	**13**	**14**		**11**	**12**	**13**	**14**
1	196.1	n.o.	203.7	204.0	1A	97.9	97.9	96.8	96.9
2	112.6	112.6	77.6	76.8	2A	36.9	36.9	37.2	37.5
3	193.1	n.o.	42.4	42.8	3A	82.0	82.1	80.4	81.4
4	77.6	77.6	27.1	27.3	4A	90.6	91.0	75.2	75.4
4-OCH_3	61.2	61.2			5A	70.5	70.5	72.3	72.6
4a	42.7	42.5	136.4	136.5	6A	12.7	12.7	17.9	17.9
5	26.5	27.0	101.7	101.7	1B	100.5	100.5	99.4	100.0
5a	134.3	133.0			2B	34.8	34.8	39.7	40.0
6	117.6	117.8	159.6	159.2	3B	68.8	68.9	70.9	71.3
7	102.3	103.0	111.1	111.1	4B	70.3	70.6	77.0	77.5
7-CH_3			7.97	7.9	5B	71.5	71.5	72.6	72.6
8	161.7	163.0	155.7	156.2	6B	16.4	16.7	17.6	16.5
8a			107.8	108.4	1C			101.3	100.9
9	110.4	102.0	164.0	165.3	2C			36.3	38.0
9a			108.7	108.6	3C			81.4	81.8
9-CH_3	7.7				4C			91.3	75.7
10	157.1	160.6	117.3	117.2	5C			73.8	72.6
10a	106.8	107.1	138.9	139.0	6C			12.7	17.6
1′	202.9	202.9	81.9	82.1	1D			100.1	100.4
1′-OCH_3			58.7	58.8	2D			34.4	36.0
2′	27.9	27.3	212.1	211.3	3D			68.7	68.9
3′			79.1	79.2	4D			69.9	70.2
4′			68.3	68.4	5D			71.5	71.1
5′			19.3	19.6	6D			16.4	14.7
					1E				97.5
					2E				48.0
					3E				69.2
					3E-CH_3				23.8
					4E				206.0
					5E				71.7
					6E				18.0

[a] n.o. = not observed.

D-oliose moiety, possibly MtmGIV (see below), is unable to recognize an unmethylated sugar donor substrate for this particular glycosyl transfer step and, therefore, seems to be inhibited when the methyl group is missing in the sugar cosubstrate.

The accumulation of products showing an unreduced C-4 carbonyl group at the first sugar moiety of the lower disaccharide chain was also proven to be the consequence of the inactivation of *mtmC*, since mithramycin biosynthesis was restored in the corresponding mutants by expressing in trans the *mtmC* gene. Since a polar effect on the neighbor gene *mtmTIII* could be ruled out, this result suggests that inactivation of *mtmC* is affecting a ketoreductase other than MtmTIII. Possible candidates are MtmTI or MtmTII, whose functions within the biosynthetic pathway of **10** have yet to be determined. Type-II PKSs are likely to be assembled in a highly structured multifunctional array resulting in a 3-dimensional structure, which might be crucial to assist folding of the growing polyketide chain as well as for the activity of certain biosynthetic enzymes. Similar unpredicted results as found here led already to the conclusion that a complex association of many proteins is sometimes necessary in type-II PKS systems and that removal of one component can have unpredictable indirect effects on the behavior of the remaining activities.[59] Thus, the modification of the spatial structure of the protein cluster by inactivation of *mtmC* may in this case be the underlying cause by which the ketoreductase responsible for the 4-reduction of the first sugar building block of the trisaccharide chain, although expressed, is rendered inactive.

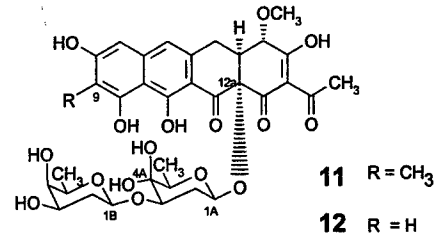
Inactivation of MtmTIII showed that this enzyme is responsible for the 4-ketoreduction step of the mycarose building block biosynthesis, considering the product isolated from the MtmTIII mutant (**14**) contains a 4-ketomycarose instead of a mycarose unit at the end of the trisaccharide chain. That MtmTIII is responsible for a ketoreduction in a sugar building block biosynthesis seemed at first somewhat surprising, since the deduced amino acid sequence of MtmTIII resembles mostly ketoreductases involved in polyketide aglycon biosyntheses. The closest resemblance was found for DnrE and DauE,[40] which catalyze an early step of the biosynthesis of anthracyclinones, namely the reduction of the tetracyclic aklaviketone to aklavinone. Only weak resemblance could be found with ketoreductases involved in deoxysugar biosyntheses. A possible explanation for this is that the reduction of the 4-keto group of this sugar moiety happens *after* its attachment. Thus, MtmTIII has to act on a large molecule, composed of a tetracyclic polyketide-derived aglycon (like aklaviketone) and an attached trisaccharide, instead of on a single NDP-deoxysugar. Transfer of biosynthetic intermediates of deoxysugars that are still unreduced at their 4-position is possible; however, it is very unusual and rarely found in antibiotic biosynthetic pathways. For example, in a recent publication, Liu and co-workers generated new methymycin/pikromycin analogues bearing modified sugars, revealing a highly relaxed sugar cosubstrate specificity of the glycosyltransferase DesVII. Despite its unusual substrate flexibility, DesVII failed to transfer 4-ketosugars.[60] In contrast,

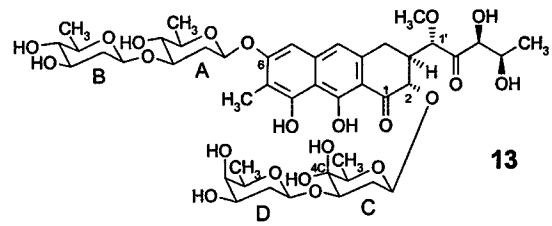
(59) Staunton, J.; Weissman, K. J. *Nat. Prod. Rep.* **2001**, *18*, 380−416.

Table 3. Long-Range Couplings Observed in the HMBC Spectra of the New Premithramycin (**11**, **12**) and Mithramycin Derivatives (**13**, **14**) (Most Important Couplings Are Highlighted in Bold)

	C-position			
H-position	**11**	**12**	**13**	**14**
2			1,3,1′,1C	3,1′,1C
3				4
4	1,4-$OCH_{3,4a,5}$			
α			4a	4a,10
β			2,3,4a,9a,10	2,4a,9a,10
4-OCH_3	4	4		
4a				
5			6,7,8,8a,9,9a,10	6,7,10
α	4a,5a			
β				
6	5,6a,7,10a,11a	5,7,11a		
7	6,8,9,10a	6,8,9,10a		
7-CH_3			5,6,7,8,8a,9a	6,7,8,10a
9		7,8,10,10a		
9-CH_3	8,9,10			
10			4,5,8a,9a,10a	4,5,8,8a,9,9a,10a
14	2,13	13		
1′			2,3,4,1′-$OCH_{3,2'}$	3,4,1′-$OCH_{3,2'}$
1′-OCH_3			1′,3′	2,1′
3′			2′,4′,5′	2′
4′			2′,5′	
5′			2′,3′,4′	3′,4′
1A	12a		6	6,2A
$2A_a$	1A,3A	1A,3A	1A,3A	1A,3A
$2A_e$	3A,4A	3A,4A	1A,3A,4A	1A,3A,4A
3A	1B	1B	2A,4A,1B	1B
4A			3A,5A,6A	3A,5A
5A	**1A,6A**	**1A,6A**	1A,3A,4A,6A	1A,4A
6A	4A,5A	4A,5A	4A,5A	5A
1B	3A	3A	3A	3A,2B
$2B_a$	1B,3B	1B,3B	1B,3B,4B	1B,3B
$2B_e$			1B,3B,4B	1B,3B,4B
3B			2B,4B,5B	4B
4B	5B	5B	3B,5B,6B	3B,5B
5B	4B,6B	4B,6B	1B,3B,4B,6B	1B,3B,4B
6B	5B	5B	3B,4B,5B	2B,5B
1C				2,2C
$2C_a$			1C,3C	1C,3C
$2C_e$			1C,3C,4C	1C,3C,4C
3C			2C,1D	4C,1D
4C				3C,5C
5C			1C,3C,4C,6C	1C,4C
6C			4C,5C	2C,4C,5C
1D			3C	3C
$2D_a$			1D,3D	1D,3D
$2D_e$			4D	
3D			2D,4D	
4D			2D,3D,5D	2D,3D
5D			1D,4D,6D	3D,4D
6D			4D,5D	
1E				2E
$2E_a$				1E
$2E_e$				1E,4E
3E-CH_3				1E,2E,3E,4E
5E				1E,4E
6E				4E,5E

an EryBIV mutant from the erythromycin producer, *Saccharopolyspora erythraea*, has been previously reported to transfer a 4-keto-L-mycarose unit to the erythronolide B aglycon.[61] Also, the GT responsible for the glycosyltransfer step, through which the D-mycarose moiety is linked during the biosynthesis of **10**, as well as the GT, which attaches the first sugar unit of the trisaccharide chain, are capable of transferring 4-ketosugars, as





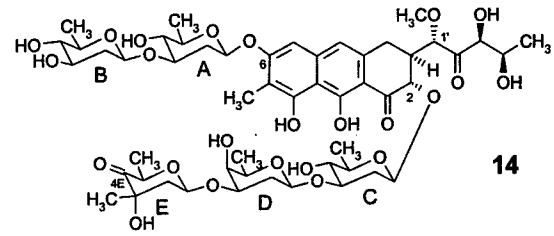


Figure 3. New structures resulting from the *mtmC*- and *mtmTIII*-inactivation experiments: 4A-ketopremithramycin A2 (**11**), 4A-keto-9-demethylpremithramycin A2 (**12**), 4C-keto-demycarosylmithramycin (**13**), and 4E-ketomithramycin (**14**).

was shown in products **11**−**13** and **14**, respectively. Since such a capability is rarely found among GTs, it seems reasonable to assume that a single GT may be responsible for both of these transfer steps and that, as suggested by the results presented above, the corresponding sugars are reduced after attachment as ketosugars. A dual role for one of the *mtm* GTs is also necessary because of the presence of five sugar moieties, but only four GT encoding genes in the *mtm* gene cluster. MtmGIV has previously been shown to be responsible for the attachment of the first sugar of the trisaccharide chain, a D-olivose, and thus is the best candidate for such a double role. As with all GTs, MtmGIV is believed to have two binding sites, one for the alcohol substrate or the acceptor substrate (here **1**) and a second for the sugar cosubstrate, the donor substrate. It seems that MtmGIV may have a unique sugar binding site, which recognizes and selectively attaches two different sugars, a 4-ketoolivose as the first sugar and a 4-ketomycarose as the last sugar of the trisaccharide chain, respectively. The change of the alcohol acceptor substrate, here in particular the absence or presence of a D-olivose-D-oliose disaccharide chain, might also influence the sugar donor binding site, thereby changing its specificity and thus accounting for the differentiation between NDP-ketoolivose and NDP-ketomycarose as sugar cosubstrates.

The experiments described here also raise important issues regarding the flexibility of other enzymes involved in the biosynthesis of **10**. For example, MtmGIII, which normally attaches D-oliose to the (already 4-reduced!) D-olivose of

(60) Yamase, H.; Zhao, L.; Liu, H.-w. *J. Am. Chem. Soc.* **2001**, *123*, in press.
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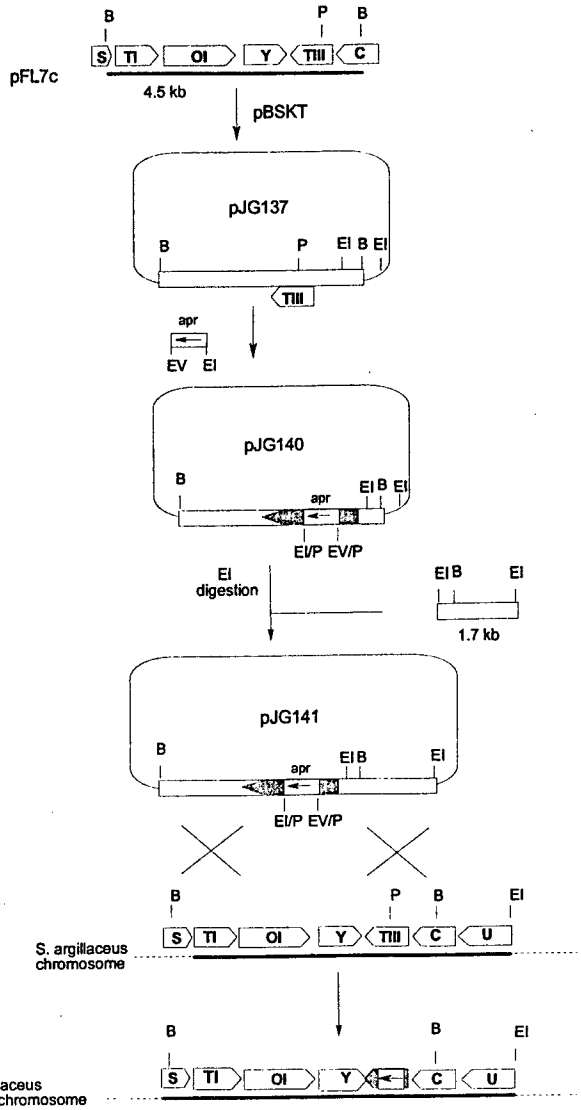

Figure 4. Scheme representing the insertional inactivation of *mtmTIII* and the replacement in the chromosome of the wild-type *mtmTIII* allele by the in vitro mutated one. B, *Bam*HI; EI, *Eco*RI; EV, *Eco*RV; P, *Pml*I; apr, apramycin resistance gene.

premithramycin A1 (**3**), shows a unique flexibility toward its acceptor substrate by attaching D-oliose also to a 4-ketosugar. To our knowledge, this is the first example in a deoxysaccharide biosynthesis in which a GT was able to link a deoxysugar to another genetically modified deoxysugar that includes a keto group neighboring the linkage position. The hydrate formation, which changes 4-keto-D-olivose to 4-hydroxy-D-olivose, may be indeed helpful for this process, since 4-hydroxy-olivose is stereoelectronically more similar to olivose than the keto form. In addition, compound **13** is the first case in which MtmGI and MtmGII have been shown to attach the disaccharide chain even though the trisaccharide chain is incomplete. It is also worth pointing out that oxygenase MtmOIV was capable of oxidatively opening the fourth ring of the **13** precursor, although this lacks the mycarose moiety and has the (hydrated) keto function in the 4C-position as compared to the natural substrate premithramycin B (**9**). Furthermore, MtmOIV was able to process the precursor of 4E-ketomithramycin (**14**) despite its keto function in the 4E-position. Along with the previously published **10** derivatives, 7-demethylmithramycin and premithramycin A4, **13** and **14** are further examples demonstrating the relatively high degree of substrate flexibility of MtmOIV. This is an important finding, since MtmOIV plays a key role in the generation of novel mithramycin derivatives by combinatorial biosynthesis due to its ability to convert biologically inactive tetracyclic premithramycins into active tricyclic mithramycins.[39]

In conclusion, the work described here on the two neighboring genes *mtmC* and *mtmTIII* reveals that the corresponding enzymes, MtmC and MtmTIII, are responsible for the 3-methyltransfer and the 4-ketoreduction, respectively, occurring during the generation of the D-mycarose moiety of **10**. Glycosyltransferase MtmGIV presumably displays a double role, likely being responsible for the linkage of both the first and the third sugar of the trisaccharide chain. These sugars appear to be attached as 4-ketosugars, and the 4-ketoreduction happens after the glycosyltransfer step. Finally, four new compounds, two of which are potentially active **10** analogues, were created as a result of the inactivation experiments. All these results also provide important additional information regarding the substrate specificity of several of the post-PKS enzymes involved in mithramycin biosynthesis.

Experimental Section

Microorganisms, Culture Conditions, and Vectors. *Streptomyces argillaceus* ATCC 12956, a mithramycin producer, was used as the source of chromosomal DNA. For sporulation it was grown for 7 days at 30 °C on plates containing A medium.[36] For protoplast regeneration, the organism was grown on R5 solid medium,[46] and R5A medium was used as the liquid medium for production.[36] *Escherichia coli* XL1-Blue[47] was used as the host for subcloning and was grown at 37 °C in TSB medium (tryptic soy broth, Oxoid). When plasmid-containing clones were grown, the medium was supplemented with the appropriate antibiotics: 5 or 50 μg/mL of thiostrepton for liquid or solid cultures, respectively, 100 μg/mL of ampicillin, 25 μg/mL of apramycin, or 20 μg/mL of tobramycin. Plasmid pBSKT[38] was the vector used for insertional inactivation experiments.

DNA Manipulation. Plasmid DNA preparations, restriction endonuclease digestions, alkaline phosphatase treatments, ligations, and other DNA manipulations were performed according to standard procedures for *E. coli* [48] and for *Streptomyces*.[46]

Insertional Inactivation of *mtmC*. A 5.5 kb *Pst*I fragment containing the *mtmC* gene was subcloned as a *Xba*I−*Hind*III fragment (using these sites from the polylinker) into the same sites of pBSKT, generating pM7C0. A *Bam*HI−*Bgl*II fragment from pUO9090, containing an apramycin resistance cassette, was then subcloned in both orientations into the unique *Bam*HI site in pM7C0 located within the *mtmC* gene, generating two different constructs: pM7C1, with the resistance gene oriented in the same direction of transcription as *mtmC*, and pM7C1R, with the resistance gene inserted in the opposite direction. These constructs were used to transform protoplasts of the wild-type strain *S. argillaceus* ATCC 12956. Transformants were selected on the basis of resistance to 25 μg/mL of apramycin. To select disruptants in which the wild-type region of the chromosome was replaced by the in vitro mutated one through a double crossover, transformant colonies were grown in TSB medium without antibiotics. After 72 h, protoplasts were obtained and plated onto a regeneration medium. Growing colonies were tested for resistance to apramycin and sensitivity to thiostrepton (50 μg/mL). The replacement was also confirmed by Southern analysis.

Complementation of the *mtmC*-Minus Mutants. The 2 kb *Sna*BI−*Pml*I fragment from pLP7 was subcloned into the *Sma*I site of pUK21, generating PAGC0. Then the *Spe*I fragment using the sites from the flanking polylinker was subcloned into the *Xba*I site of pIAGO, in the correct orientation and downstream of the erythromycin resistance

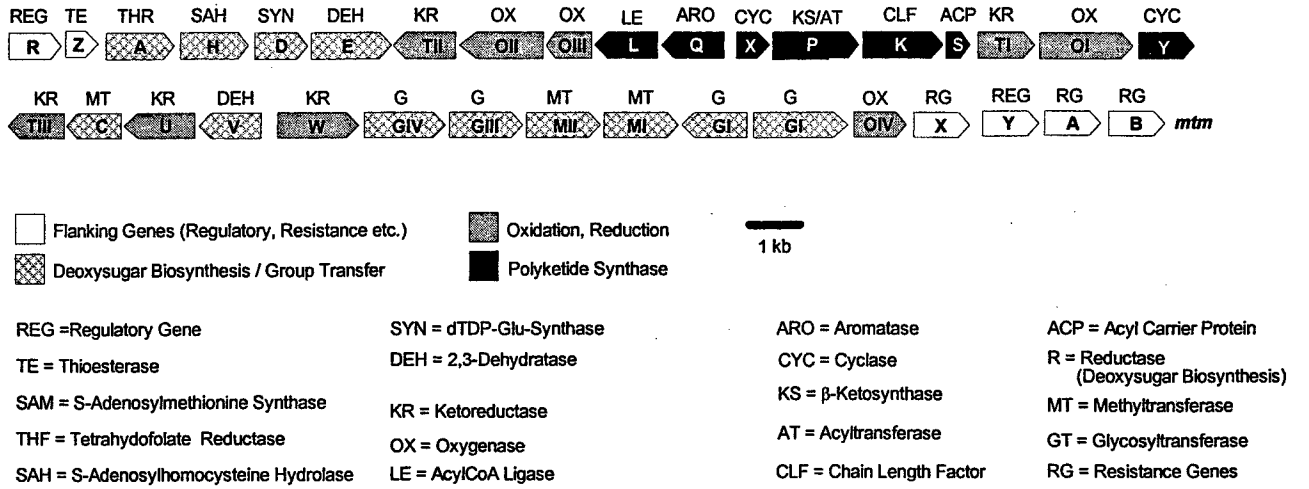

Figure 5. Genetic organization of the mithramycin gene cluster.

promoter, generating the multicopy plasmid pAGC1. Protoplasts of mutants M7C1 and M7C1R were transformed with pAGC1.

Insertional Inactivation of *mtmTIII*. A 4.5 kb *Bam*HI fragment containing the *mtmTIII* gene was subcloned into the *Bam*HI site of pBSKT. In this construct, an apramycin resistance cassette was subcloned as an *Eco*RI−*Eco*RV (this site blunt-ended) fragment into the unique *Pml*I site which is located within the *mtmTIII* coding region. This construct (pJG140) was digested with *Eco*RI, and a 1.7 kb *Eco*RI fragment was inserted to produce pJG141, in which the right-hand side of the insert was enlarged with additional chromosomal DNA from that region to facilitate the occurrence of a crossover. This final construct (pJG141) was used to transform protoplasts of the wild-type strain *S. argillaceus* ATCC 12956. Transformants in which the replacement of the wild-type region of the chromosome was replaced by the in vitro mutated one through a double crossover at both sides of the apramycin cassette were recognized by resistance to apramycin and sensitivity to thiostrepton (Figures 4 and 5). The replacement was also verified by Southern analysis.

Isolation, Purification, and HPLC of the New Compounds. Detection of mithramycin-related compounds was performed on a reversed phase column (Symmetry C18, 4.6 × 250 mm, Waters), with acetonitrile and water supplemented with 0.1% trifluoroacetic acid as solvents (linear gradient from 10 to 100% acetonitrile in 30 min, at a flow rate of 1 mL/min). Detection and spectral characterization of peaks were accomplished with a photodiode array detector and Millennium software (Waters), extracting bidimensional chromatograms at 280 nm. To isolate the new compounds, spores of the mutants were initially grown in TSB medium during 24 h at 30 °C and 250 rpm. This seed culture was used to inoculate (at 2.5%, v/v) eight 2 L Erlenmeyer flasks containing 400 mL of R5A medium. After incubation for 4 days at the above conditions, the cultures were centrifuged, filtered, and extracted as described.[36] Mithramycin-related compounds (identified according to their spectral characteristics) were purified by preparative HPLC on a μBondapak C18 radial compression cartridge (PrepPak Cartridge, 25 × 100 mm, Waters). Short gradients using 0.1% trifluoroacetic acid in water and either methanol or acetonitrile, at 10 mL/min, were optimized for resolution of individual peaks. The purified material collected in each case was diluted 4-fold with water, applied to a solid-phase extraction column (Lichrolut RP-18, Merck), washed with water to eliminate trifluoroacetic acid, eluted with methanol, and dried in vacuo. Yields: **11**, 12 mg/L; **12**, 10 mg/L; **13**, 3 mg/L; **14**, 12 mg/L (for comparison reasons; the production of **10** by the wild-type strain of *S. argillaceus* is 10−12 mg/L).

Structure Elucidation of 4A-Ketopremithramycin A2, 4A-Keto-9-demethylpremithramycin A2, 4C-Keto-demycarosylmithramycin, and 4E-Ketomithramycin. The structures of new premithramycin and mithramycin derivatives were elucidated by NMR and mass spectroscopy. The positive fast atom bombardment (FAB) and high-resolution (HR-FAB) mass spectra were acquired at the University of South Carolina, Department of Biochemistry and Chemistry facilities in Columbia, SC, using a VG70SQ double focusing magnetic sector MS instrument. The atmospheric pressure chemical ionization mass spectra (APCI-MS) were acquired at the Medical University of South Carolina, Regional Mass Spectroscopy Center, using a Finnigan MAT LCQ. All the NMR data were performed in d_6-acetone/D_2O (95:5), either on a Varian Inova 400 or a Bruker DMX 500 instrument. The NMR data are listed in Tables 1 and 2; the important long-range C−H couplings observed in the HMBC spectra are shown in Table 3. All NMR assignments are based on DEPT, H,H-COSY, HSQC, TOCSY, and HMBC spectra,[37,49−53] allowing an unambiguous assignment of all NMR signals.

Acknowledgment. This work was supported by grants of the South Carolina Commission of Higher Education (2000−2001), the U.S. Department of Defense, and the National Institutes of Health (R01CA91901) to J.R., and by the Spanish Ministry of Education and Science to J.A.S. through the "Plan Nacional en Biotecnologia" (BIO97-0771). Drs. William Cotham and Michael Walla, Department of Chemistry and Biochemistry, University of South Carolina, are acknowledged for recording the FAB mass spectra.

JA0105156

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Published on Web 05/04/2002

Rationally Designed Glycosylated Premithramycins: Hybrid Aromatic Polyketides Using Genes from Three Different Biosynthetic Pathways

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Received October 25, 2001

Abstract: Heterologous expression of the *urdGT2* gene from the urdamycin producer *Streptomyces fradiae* Tü2717, which encodes a *C*-glycosyltransferase, into mutants of the mithramycin producer *Streptomyces argillaceus*, in which either one or all glycosyltransferases were inactivated, yielded four novel *C*-glycosylated premithramycin-type molecules. Structure elucidation revealed these to be 9-*C*-olivosylpremithramycinone, 9-*C*-mycarosylpremithramycinone, and their respective 4-*O*-demethyl analogues. In another experiment, both the *urdGT2* gene from *S. fradiae* and the *lanGT1* gene from *S. cyanogenus*, were coexpressed into a *S. argillaceus* mutant lacking the MtmGIV glycosyltransferase. This experiment, in which genes from three different organisms were combined, resulted in the production of 9-*C*-(olivo-1−4-olivosyl)premithramycinone. These results prove the unique substrate flexibility of the *C*-glycosyltransferase UrdGT2, which tolerates not only a variety of sugar-donor substrates, but also various acceptor substrates. The five new hybrid products also represent the first compounds, in which sugars were attached to a position that is normally unglycosylated. The successful combination of two glycosyltransferases in the latter experiment proves that the design of saccharide side chains by combinatorial biosynthetic methods is possible.

Introduction

Polyketides constitute a large and structurally diverse family of pharmaceutically important natural products.[1] Many polyketides have useful biological activities, and they have found clinical applications as antibiotics, antifungals, antiparasitics, anticancer, or immunosuppressant agents and also as herbicides and insecticides.[2] Although the chemical structures of polyketides are very diverse, all of them are assembled in a similar manner. The biosynthesis of the polyketide skeleton occurs through the condensation of short-chain carboxylic acids in a series of reactions catalyzed by polyketide synthases.[3] This condensation process generates carbon chains of varying length, with different side chains and reduction patterns that are differentially cyclized and subsequently modified to give the final structures. A number of polyketide structures contain sugar moieties attached to the aglycons. These sugar components usually participate in the molecular recognition of the cellular target, and they are therefore important, and often essential, for the biological activity.[4] Most of these sugars belong to the wide family of the 6-deoxyhexoses, which to date comprises more than 70 different deoxyhexoses, identified in a variety of natural product pathways.[5] Due to the importance of the sugars for biological activity, the idea has emerged of generating novel glycosylated derivatives by altering the glycosylation pattern of bioactive compounds. Recent evidence increasingly suggests some degree of flexibility of glycosyltransferases involved in the biosynthesis of secondary metabolites, and some examples have been reported in which foreign sugars have been transferred to aglycons.[6] In all of these examples, the attachment of the new

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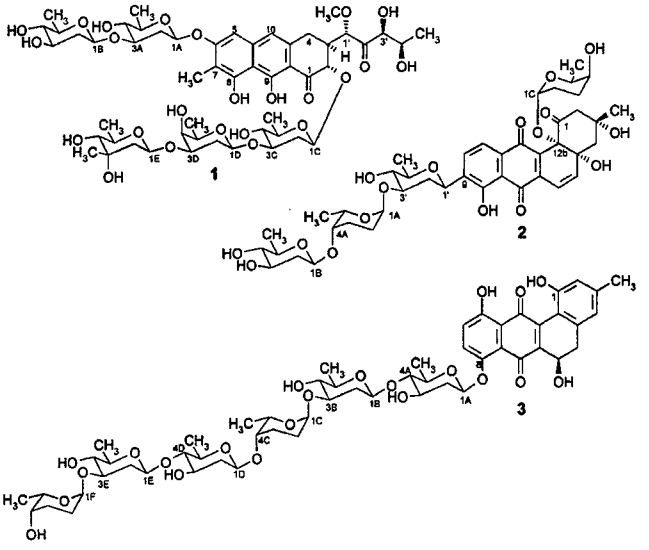
10.1021/ja017385l CCC: $22.00

sugars took place at the same position of the aglycon as the natural sugar. However, as far as we know, there are no reports in the literature of secondary metabolism glycosyltransferases being used to attach a sugar moiety to a different position of the aglycon.

Here we report the rational design of novel glycosylated derivatives from two nonglycosylated tetracyclic premithramycin-type molecules (4-demethylpremithramycinone and premithramycinone) by expressing a glycosyltransferase gene from the urdamycin A pathway of *Streptomyces fradiae* into mithramycin nonproducing mutants of *S. argillaceus*. In addition, we also report the extension of this glycosyl moiety into a disaccharide chain by expressing another glycosyltransferase gene from the landomycin A pathway of *S. cyanogenus*. The latter experiment results in an aromatic hybrid polyketide which derives from a combination of genes from three different pathways.

Mithramycin (**1**), an aromatic polyketide produced by *S. argillaceus* ATCC 12956, is used for the treatment of certain cancers, such as testicular carcinoma or disseminated embryonal cell carcinoma as well as for the treatment of Paget's disease and to control hypercalcemia in patients with malignant diseases.[7a,b] Structurally, it is composed of a tricyclic aglycon with a C_5-side chain, a dissaccharide (D-olivose−D-olivose) located at the 6-position and a trisaccharide (D-olivose−D-oliose−D-mycarose), attached to the 2-position.[7c] It has been shown that the glycosylation steps of the biosynthesis of mithramycin occur on tetracyclic intermediates. First, the sugars of the trisaccharide are sequentially added to the premithramycinone aglycon. Then a previously formed D-olivosyl-D-olivose disaccharide is transferred intact into the pseudoaglycon.[8b,e] As one of the last steps in the mithramycin biosynthesis, an oxidative cleavage of the fourth ring of the fully glycosylated tetracyclic intermediate premithramycin B results in a tricyclic compound, which is the immediate precursor of mithramycin.[8] (Figure 1). A number of these tetracyclic biosynthetic intermediates of mithramycin have been isolated from different mutants and their structures have been elucidated.[6j,8] Two of them (4-demethylpremithramycinone and premithramycinone) contain no sugar moieties, others possess mono-, di-, or trisaccharides at the 6-position (premithramycins A1, A2, and A3).



Urdamycin A (**2**) is an angucycline polyketide produced by *S. fradiae* Tü2717 which also shows antitumor activity.[9] It consists of the aglycon aquayamycin, which includes a *C*-glycosidically linked D-olivose, and three additional *O*-glycosidically linked deoxyhexoses: two L-rhodinoses and another D-olivose. Four glycosyltransferase genes have been identified in the urdamycin gene cluster and specific functions have been assigned to each of them.[10] The UrdGT2 glycosyltransferase catalyzes the earliest glycosylation step in the urdamycin biosynthesis. This *C*-glycosyl transfer step requires an activated D-olivose as sugar donor substrate and an unknown angucyclinone as the acceptor substrate, possibly compound **4**, the product being compound **5**.[10a] The putative substrate (**4**) of UrdGT2 resembles to some extent the intermediates of the mithramycin biosynthetic pathway, particularly 4-demethylpremithramycinone (**6**) and premithramycinone (**7**, Figure 2). Thus, we anticipated that UrdGT2 might recognize and glycosylate these intermediates of the mithramycin biosynthetic pathway and thereby create novel glycosylated derivatives.

Results and Discussion

To test this hypothesis we expressed *urdGT2* under the control of the erythromycin resistance promoter (*ermE**p) in several mutants of *S. argillaceus* that accumulate different tetracyclic intermediates. The selected mutants had been generated by gene replacement of all four mithramycin glycosyltransferase genes (M3ΔMG mutant) or by gene replacements of single genes

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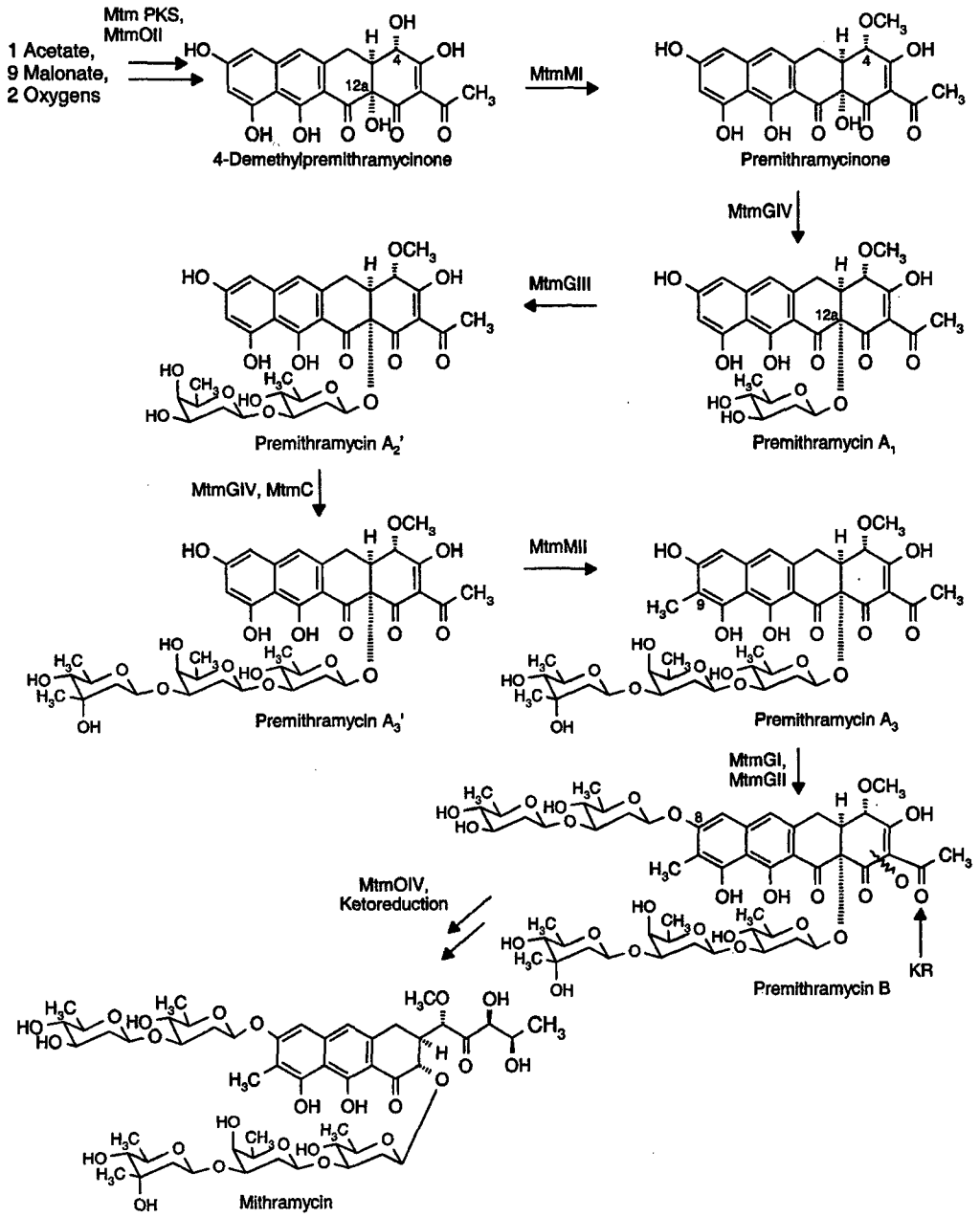

Figure 1. Late biosynthetic pathway to mithramycin showing the sequence of events of the post-PKS tailoring steps catalyzed by oxidoreductases (O = oxygenase; KR = ketoreductase) or group transferases (G = glycosyltransferase; M = methyltransferase). The newly added structural elements of each shown step are highlighted in bold.

encoding glycosyltransferases (strains M3G1, M3G2, M3G3, and M3G4 with mutations in *mtmGI*, *mtmGII*, *mtmGIII*, and *mtmGIV*, respectively).[8a−e] Expression of *urdGT2* in mutants M3G1, M3G2 and M3G3 did not lead to the production of any new compound as shown when cultures were extracted with ethyl acetate and analyzed by reverse phase HPLC. However, upon expression of *urdGT2* in M3ΔMG and M3G4 mutants, four new HPLC-peaks (two in each mutant) were observed with the characteristic absorption spectra of the tetracyclic premithramycin-type aglycons. The new compounds were isolated by preparative HPLC and their structures were elucidated using NMR and mass spectroscopy.

Mutant M3G4(*urdGT2*) yielded two compounds. Both compounds exhibit all of the typical NMR signals of a tetracyclic premithramycin-type aglycon, except for the 9-position. In all previously isolated molecules of such type, this position is substituted by either a proton, as in premithramycinone[8c] (**7**) and premithramycin A1[8b] (**8**), or by a methyl group, for example as in premithramycin A2[8b] (**9**) or premithramycin A3[8b] (**10**). The ^{1}H NMR data (Tables 1, 2) of the new compounds showed neither a 9-H nor a 9-CH_3 signal. Such a methyl group was also not observed in the ^{13}C NMR spectra, which revealed C-9 to be a quaternary carbon. This is all consistent with structures in which the 9-position is substituted by a different residue. Since the NMR data also indicated the presence of one deoxyhexose moiety in each of the compounds, a linkage of these deoxysugar moieties at 9-position seemed likely. Indeed, the ^{13}C NMR signals of C-9 (δ_C 111.5/111.4) and for C-1A (δ_C 72.5/70.2), the anomeric carbons of the sugar moieties, show the absence of a directly bound oxygen atom at C-9 and only

Table 1. ^{1}H NMR Data of 9-*C*-Olivosylpremithramycinone (**12**), 9-*C*-Mycarosylpremithramycinone (**14**), 9-*C*-Olivosyl-4-*O*-demethylpremithramycinone (**11**), 9-*C*-Mycarosyl-4-*O*-demethylpremithramycinone (**13**), and 9-*C*-Di-olivosylpremithramycinone (**15**) in Acetone-d_6 at 400 MHz,[a] δ in ppm Relative to Internal TMS

	δ, multiplicity (*J*/Hz)				
position	**12**[b]	**14**[c]	**11**[d]	**13**[e]	**15**[e,g]
4-H	4.18 br d (11.5)	4.18 br d (11.5)	4.42 br m	4.42 br m	4.24 br d (11.5)
4-OCH_3	3.59 br s	3.58 br s	-	-	3.58 br s
4a−H	2.68 br d (11.5)	2.72 br d (11.5)	2.70 ddd (11.5, 5, 3)	2.70 ddd (11.5, 4.5, 3)	2.72 ddd (11.5, 4.5, 3)
5-H	a: 3.48 ddd (16, 5, 2)	a: 3.48 ddd (16, 5, 2)	a: 3.45 ddd (16, 5, 1)	a: 3.45 ddd (16, 4.5, 1)	a: 3.49 ddd (16, 4.5, 1.5)
	e: 3.15 dd (16, 3)	e: 3.14 dd (16, 3)	e: 3.25 dd (16, 3)	e: 3.25 dd (16, 3)	e: 3.16 dd (16, 3)
6-H	7.00 br s	7.00 br s	6.99 br s	6.98 br s	7.02 br s
7-H	6.62 s	6.60 s	6.62 s	6.62 s	6.68 s
2′-H_3	2.61 s	2.62 s	2.61 s	2.63 s	2.62 s
1A-H	5.30 dd (12, 2)	5.62 dd (12, 2)	5.30 dd (12, 2)	5.62 dd (12, 2)	5.30 dd (12, 2)
2A-H	a: 1.80 ddd (13, 12, 12)	a: 1.86 dd (13, 12)	a: 1.80 dd (13, 12)	a: 1.85 dd (13, 12)	a: 1.76 ddd (13, 12, 12)
	e: 2.21 ddd (13, 5, 2)	e: 1.99 dd (13, 2)	e: 2.20 ddd (13, 5, 2)	e: 2.00 dd (13, 2)	e: 2.25 ddd (13, 5, 2)
3A-H	3.73 ddd (12, 9, 5)	-	3.73 ddd (12, 9, 5)	-	3.71 ddd (12, 9, 5)
3A-CH_3	-	1.26 s	-	1.25 s	-
4A-H	3.18 dd (9, 9)	3.23 br d (9.5)	3.17 dd (9, 9)	3.21 br d (9.5)	3.30 dd (9, 9)
5A-H	3.56 dq (9, 6.5)	3.84 dq (9.5, 6)	3.57 dq (9, 6.5)	3.83 dq (9.5, 6)	3.62 dq (9, 6.5) obsc
6A-H_3	1.40 d (6.5)	1.38 d (6)	1.40 d (6.5)	1.38 d (6)	1.41 d (6.5)

[a] Recorded at 500 MHz. br = broad; obsc = obscured by solvent or water. [b] OH signals (not assigned): 4.20, 4.30, 5.70 (3′-OH), 9.30, 10.20, 15.58, 18.18. [c] OH signals (not assigned): 3.60, 4.02, 5.70, 9.40, 10.00, 16.60, 18.20. [d] OH signals (not assigned, not all observed): 5.75, 9.30, 10.00. [e] OH signals (not assigned, not all observed): 5.80, 9.40, 9.98. [f] OH signals (not assigned, not all observed): 4.32, 4.58, 5.72, 9.13, 10.08. [g] Signals of the second olivose moiety: 4.75 dd (1B-H, J = 12, 2); 1.56 ddd (2B-H_a, J = 13, 12, 12); 2.25 ddd (2B-H_e, J = 13, 5, 2); 3.60 m obsc (3B-H); 2.99 dd (4B-H, J = 9, 9); 3.40 dq (5B-H, J = 9, 6.5); 1.29 d (6B-H_3, J = 6.5).

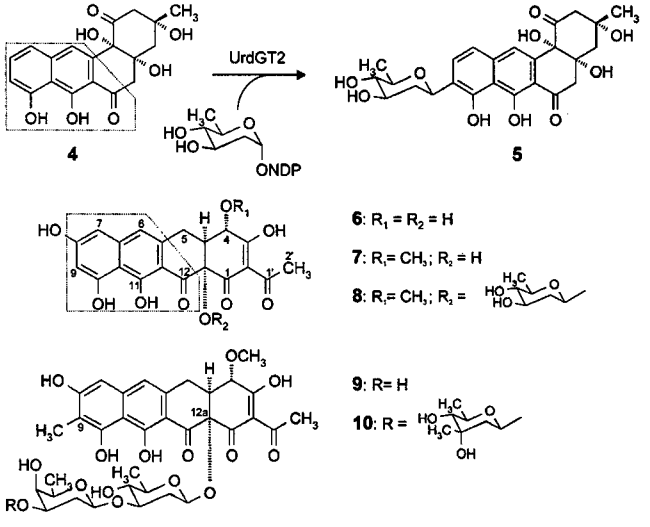

Figure 2.

one directly attached oxygen atom at the anomeric carbon C-1A, respectively. This clearly indicates a *C*-glycosidic linkage of the sugar building blocks at the 9-position. The sugar moiety of the major compound is olivose, as indicated by the H,H coupling constants, along with the other ^{1}H- and ^{13}C NMR data (see tables 1, 2), and mycarose for the minor compound. Representative signals for the latter are the 3A-methyl group (δ_H 1.26, δ_C 27.6) instead of a 3A-H signal, along with the simpler ^{1}H NMR signals for 2A-H and 4A-H, which both lack one a,a-coupling compared to the respective patterns in olivose. Both sugar moieties are β-glycosidically linked, as revealed by the large (12 Hz) coupling constants of the anomeric protons which appear at δ 5.30 (dd, J = 12 and 2 Hz) and δ 5.62 (dd, J = 12 and 2 Hz), respectively. Thus, the two compounds of mutant M3G4(*urdGT2*) are 9-*C*-olivosylpremithramycinone (**12**) and 9-*C*-mycarosylpremithramycinone (**14**). The yields for these compounds were 5.2 mg/L for **12** and 5.8 mg/l for **14**. The deduced molecular formula for the former compound is $C_{27}H_{28}O_{12}$ (544.5), which was confirmed by the APCI mass spectrum (*m/z* 543, M − H$^-$,100%). In conformity with structure **14**, the APCI mass spectrum shows a molecular ion at *m/z* 557 (M − H$^-$, 100%), proving the molecular formula of $C_{28}H_{30}O_{12}$ (558.5).

Compared with these two compounds, the two minor compounds found in mutant M3ΔMG(*urdGT2*) are almost identical in respect to all NMR data. Again, one of these minor compounds contains an olivose moiety, while the other contains a mycarose moiety. They differ from compounds **12** and **14** only in that they possess a 4-OH instead of a 4-*O*-methyl group. This is revealed in the NMR spectra, where the *O*-methyl signal (in compounds **12** and **14** at δ_H ca. 3.6/δ_C ca. 62) is missing. In agreement with this, the 4-H signals of both additional compounds shifted downfield from ca. δ 4.18 to δ 4.42, and the 4-H signals appear as a broad multiplet instead of as a broad doublet due to the additional H,OH coupling. A smaller downfield shift of 0.1 ppm can also be observed on the neighboring 5-H_e protons. Otherwise, the NMR data are almost identical with the corresponding 4-methyl ether analogues **12** and **14** (see Tables 1, 2). Thus, the NMR data allow us to suggest 9-*C*-olivosyl-4-*O*-demethylpremithramycinone (**11**) and 9-*C*-mycarosyl-4-*O*-demethylpremithramycinone (**13**) as the structures of these two minor compounds of mutant strain M3ΔMG-(*urdGT2*) with yields of 3.5 mg/L for **11** and 3.9 mg/L for **13**. As in the corresponding 4-methyl ether analogues, the sugar moieties in **11** and **14** are β-glycosidically linked, as can be deduced from the large (12 Hz) couplings constants of the anomeric protons (see Table 1).

The results prove two important facts about UrdGT2. This *C*-glycosyltransferase is not only capable of handling its normal sugar substrate NDP− D-olivose, but also NDP− D-mycarose, which is quite different from NDP− D-olivose with respect to its 3-methyl branch and in possessing an axial OH group instead of an equatorial one. In addition, these experiments illustrate again the interestingly wide substrate range of UrdGT2 regarding its acceptor substrate. Although it was noticed before that UrdGT2 can glycosylate a variety of angucyclinones and even the bicyclic quinone naphthazarine,[10a] the highly substituted

Table 2. ^{13}C NMR Data of 9-*C*-Olivosylpremithramycinone (**12**), 9-*C*-Mycarosylpremithramycinone (**14**), 9-*C*-Olivosyl-4-*O*-demethylpremithramycinone (**11**), 9-*C*-Mycarosyl-4-*O*-demethylpremithramycinone (**13**), and 9-*C*-Di-olivosylpremithramycinone (**15**) in Acetone-d_6 at 100.6 MHz, δ in ppm

	δ						δ				
position	**12**	**14**	**11**	**13**	**15**[a,b]	position	**12**	**14**	**11**	**13**	**15**[a,b]
1	197.2	197.0	n.o.	n.o.	197.2	12	191.8	191.2	192.0	192.0	191.0
2	112.0	112.0	111.0	112.2	111.4	12a	79.8	77.8	n.o.	n.o.	77.4
3	196.0	196.3	196.0	n.o.	196.0	1′	204.5	204.2	204.0	n.o.	204.6
4	77.3[1]	77.2[3]	78.0[4]	78.0	78.0	2′	28.0	28.0	28.0	28.0	28.2
4-OCH_3	61.7	62.0	–	–	61.8	1A	72.5	70.2[2]	72.5	70.3[5]	73.5
4a	44.8	44.8	46.0	46.0	45.0	2A	39.3	43.7	39.3	43.8	38.8
5	26.5	26.0	26.5	26.0	26.0	3A	78.0[1]	75.0[3]	78.0[4]	75.0	70.5
5a	135.3	135.0	136.0	135.0	135.5	3A-CH_3	~	27.6	~	27.5	?
6	118.3	118.1	118.5	118.3	118.2	4A	78.1[1]	77.3[3]	78.1[4]	77.2	88.0
6a	141.7	141.4	141.5	141.5	141.5	5A	74.2	72.0[2]	74.0	72.0[5]	76.0
7	105.0	104.9	105.0	104.8	105.0	6A	18.4	18.5	18.5	18.5	18.0
8	162.0	162.1	162.0	162.0	162.0	1B	–	–	–	–	102.0
9	111.5	111.4	111.5	111.3	110.9	2B	–	–	–	–	40.3
10	156.0	156.0	n.o.	n.o.	156.0	3B	–	–	–	–	71.3
10a	107.0	107.0	n.o.	107.0	107.0	4B	–	–	–	–	77.5
11	168.8	168.4	n.o.	n.o.	167.2	5B	–	–	–	–	72.7
11a	108.0	107.8	108.0	107.9	108.2	6B	–	–	–	–	17.9

[a] Assignments confirmed by HMBC/HSQC; n.o. = not observed. [1,2,3,4] assignments interchangeable. [b] Recorded at 125.7 MHz.

tetracyclic premithramycinone and its 4-demethyl analogue differ in their shape more drastically from the natural acceptor substrate than anything previously shown to be a substrate of UrdGT2. The fact that only premithramycinone and 4-demethylpremithramycinone glycosylated derivatives were isolated suggests that other mithramycin biosynthetic intermediates, such as premithramycin A1 (**8**), the principal product of mutant M3G3, are not good substrates for UrdGT2. Probably, the D-olivose moiety, which is attached at 12a-position in premithramycin A1,[8e] modifies the acceptor substrate to be recognizable by UrdGT2. On the other hand MtmGIV, the glycosyltransferase that catalyzes the 12a-*O*-glycosylation of premithramycinone to yield premithramycin A1,[8e] might be inhibited once the *C*-sugar unit has been linked to the 9-position (by UrdGT2).

The ability of UrdGT2 to transfer either a D-olivose or a D-mycarose moiety to the two nonglycosylated premithramycinones prompted us to design new experiments in which the possibility of extending the saccharide chain of the new compounds could be assayed. For this purpose we chose the M3G4 mutant as host and a GT-encoding gene of the landomycin A gene cluster. Landomycin A (**3**), which is produced by *S. cyanogenus* S136, contains an unusual hexasaccharide side chain consisting of four D-olivose and two L-rhodinose residues.[11] Four glycosyltransferase genes have been found in the landomycin cluster.[11d] LanGT1 was identified to be responsible for the linkage of the second sugar, a D-olivose, to the 4-OH group of the first sugar moiety of the hexasaccharide chain, which is also a D-olivose. In addition, it has been shown recently that LanGT1 displays some degree of acceptor substrate flexibility, since it was able to transfer a D-olivose moiety to the 4-position of the *C*-glycosidically linked D-olivose moiety of aquayamycin.[10d] Because of this flexibility, the *lanGT1* gene was chosen for the chain extension experiments.

Thus, the *lanGT1* gene was cloned downstream of *urdGT2* gene so that both genes were under the control of a unique *ermE** promoter. When they were coexpressed in mutant M3G4 and cultures of recombinant clones analyzed by HPLC, it was found that the peak corresponding to 9-*C*-olivosylpremithramycinone greatly decreased in favor of a new peak, while that corresponding to 9-*C*-mycarosylpremithramycinone did not change. The new compound was isolated and its structure determined by NMR and mass spectroscopy.

In contrast to the monoglycosylated structures obtained in the experiments described above, the NMR data of the new compound produced by mutant strain M3G4(*urdGT2*,*lanGT1*) clearly show the presence of two sugar moieties. The NMR data (Tables 1, 2) also indicate a premithramycinone moiety with a 4-*O*-methyl group. The exact analysis of the H,H coupling constants reveals that both sugar moieties are olivoses, one of which is *C*-glycosidically linked, the other *O*-glycosidically linked, as can be easily determined from the ^{13}C NMR shifts of the anomeric carbons (δ_C 73.5 and 102.0, respectively). The ^{13}C NMR chemical shift of C-9 (δ_C 110.9), and the fact that it is again a quaternary carbon, indicate the linkage of one of the olivoses at this position as a *C*-glycosidic moiety. This was further proven through the $^2J_{C-H}$ and $^3J_{C-H}$ long-range couplings between the anomeric proton of this olivose and carbons 8, 9, and 10 of the premithramycinone moiety observable in the HMBC spectrum. The HMBC spectrum also shows $^3J_{C-H}$ couplings between 4A-H and C-1B as well as between 1B-H and C-4A (Figure 3), which proves that the second olivose is connected to the 4A-OH group of the *C*-glycosidically linked olivose. Again the large coupling constants of the anomeric protons of both sugar moieties (both, $J_{1A-H/2A-Ha}$ and $J_{1B-H/2A-Ha}$, are 12 Hz) indicate a β-glycosidic linkage. Thus, the structure of the new compound detected in strain M3G4(*urdGT2*,*lanGT1*) is 9-*C*-(olivo-1-4-olivosyl)premithramycinone, that is, 9-*C*-diolivosylpremithramycinone (**15**). Its molecular formula $C_{33}H_{38}O_{15}$ (674.6) is confirmed by the APCI mass spectrum, which shows a molecular ion at *m/z* 674 (M^-, 70%) and some fragments. Since 9-*C*-diolivosylpremithramycinone (**15**) is the compound expected to result from the experiments as designed, it can be viewed as a rationally engineered hybrid compound.

The antitumor activities of the four compounds described here, 9-*C*-olivosylpremithramycinone, 9-*C*-mycarosylpremith-

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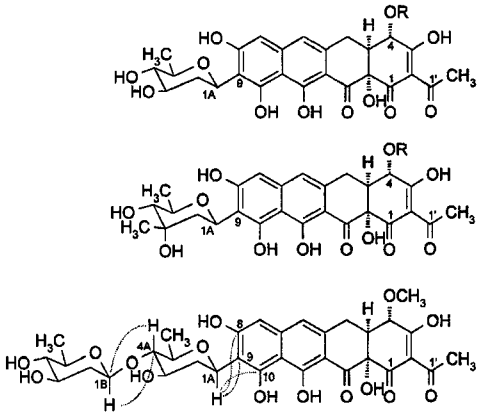

Figure 3.

ramycinone and their 4-demethylanalogues were evaluated against different tumor cell lines. Most of them showed very low activity, with 9-*C*-mycarosylpremithramycinone giving the best results. Growth percentages ranged from 52 to 110% with respect to 100% for the controls.[12]

Relaxed specificity for sugar cosubstrates has already been described for various glycosyltransferases. Recently, OleG2, an L-olivosyl transferase from *S.antibioticus*, was shown to be able to transfer L-rhamnose.[6f,h,13] TylM2, a glycosyltransferase from *S. fradiae*, was integrated into the chromosome of a triple mutant of *Saccharopolyspora erythraea*, which produced new hybrid antibiotics when the resulting strain was fed with tylactone.[15]

By deleting the gene encoding an *N*-methyltransferase contributing to the biosynthesis of D-desosamine and by recombining the D-desosamine genes with deoxysugar biosynthesis genes from the calicheamicin producer strain, it was also shown that glycosyltransferase DesVII is capable of recognizing and processing sugar substrates other than TDP-D-desosamine in the methmycin/neomethymycin pathway.[6d,15] Moreover, recombination of the desosamine genes with genes from the streptomycin producer showed that DesVII can also transfer L-sugars.[16]

The redesign and expression in *S. lividans* of deoxysugar genes of the avermectin producer *S. avermitilis* in *S. lividans* and combination with exogenously fed avermectin aglycon led to novel avermectin derivatives and showed that the *S. avermitilis* glycosyltransferase exhibits some flexibility toward the activated sugar-cosubstrate.[17] Another well characterized flexible glycosyltransferase is ElmGT from *S. olivaceus*, which is able to transfer a broad variety of L- and D-deoxysugars as well as a D-olivose disaccharide.[6a,c,i]

Glycosyltransferases that are flexible toward the alcohol acceptor substrate have also been described. For instance, expression of an *Antirhinum majus* UDP-glucose:flavonoid-3-*O*-glucosyltransferase transgene alters the flavonoid glycosylation in the lisianthus plant *Eustoma grandiflorum*.[18] Glycosyltransferase DesVII has the ability to catalyze the transfer of D-desosamine to both the 12- and 14-membered ring macrolactones of the picromycin/methymycin pathway.[19] Moreover, a D-desosamine containing polyketide library was generated by coexpression of D-desosamine biosynthetic genes, a glycosyltransferase gene from the picromycin/methymycin biosynthetic gene cluster and genes encoding genetically modified polyketide synthases in *S. lividans*.[20] These experiments show that the pikromycin desosaminyl transferase is quite tolerant of changes in the macrolactone acceptor substrate.

The glycosyltransferase UrdGT2 reported in this study showed relaxed specificity for both the sugar donor substrate and the acceptor substrate. Thus, UrdGT2 can be viewed as a very flexible enzyme with a high potential for generating novel *C*-glycosylated compounds. The coexpression of *urdGT2* and *lanGT1* resulting in the successful generation of a new compound adds another example of the design of novel compounds by combinatorial biosynthesis. In addition, it is a clear demonstration that altering the glycosylation pattern can be a convenient tool for increasing structural biodiversity from natural products.

Glycosyltransferase MtmGIV is responsible for the linkage of the first sugar moiety of the trisaccharide to the 12a-position of premithramycinone, thus converting premithramycinone (**7**) into premithramycin A1 (**8**).[8b,e] The fact that we could not detect 9-*C*-glycosylated products in all experiments where mutant strains were used that still contained the natural glycosyltransferase MtmGIV (strains M3G1, M3G2, and M3G3) of *S. argillaceus* suggests that possibly UrdGT2 cannot *C*-glycosylate the 9-position once a sugar is attached at the 12a-position. This is not really surprising, since in this situation the heterologously expressed glycosyltransferase UrdGT2 has to compete with a natural glycosyltransferase MtmGIV for the same substrate, premithramycinone (**7**). In addition, UrdGT2 also has to compete with MtmMII for the 9-position of the premithramycins. MtmMII is the methyltransferase, which in the natural mithramycin biosynthetic pathway C-methylates this 9-position.[8f] Although this step preferentially happens after completion of the trisaccharide chain, MtmMII was proven to be quite flexible in respect of its substrate, and can convert mono-, di- and triglycosylated premithramycins into their 9-methylated analogues.[8f]

The fact that no *C*-glycosylated (tricyclic) mithramycin-type molecules were found also suggests that the 9-*C*-glycosylated compounds are not good substrates for the ring-opening oxygenase MtmOIV.[8d]

Experimental Section

Microorganisms, Culture Conditions, and Plasmids. Mithramycin nonproducing mutants were used as transformation hosts: *S. argillaceus* M3ΔMG,[8d] M3G1 and M3G2,[8b] and M3G3 and M3G4.[8e] *Escherichia coli* XL1blue (Stratagene, Germany) was used as host for plasmid propagation. For sporulation on solid medium, they were grown at 30 °C on plates containing A medium.[8b] For protoplast transformation the

(12) Antitumor tests were evaluated in the three cell line system (lung NCI-H460, breast MCF7, and CNS SF-268), one dose primary anticancer assay by the National Cancer Institute, Bethesda, MD. For details, see http://dtp.nci.nih.gov.

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mutants were grown in YEME medium containing 17% sucrose. For growth in liquid medium, the organism was grown in TSB medium (trypticase soya broth, Oxoid). When plasmid-containing clones were grown, the medium was supplemented with the appropiate antibiotics: 50 μg/mL thiostrepton or 20 μg/mL tobramycin. Plasmids pMUN-*urdGT2*, pUWL-*urdGT2*, and pMUN-*lanGT1* have been previously described.[10d] Plasmid pUWL-*urdGT2-lanGT1* has been constructed during this work as described in ref 10d.

DNA Manipulation Techniques. Plasmid DNA preparations, restriction endonuclease digestions, alkaline phosphatase treatments, ligations and other DNA manipulations were according to standard procedures for *Streptomyces*.[21]

Isolation of New Compounds. Culture conditions and HPLC procedures for the purification of new compounds were as previously described.[8f]

Structure Elucidation and Characterization. NMR spectra were recorded in d_6-acetone/D_2O (95:5) on Varian Inova 400 and Bruker DMX 500 NMR instruments at 400 and 500 MHz, respectively, for ^1H and 100.6 and 125.7 MHz, respectively, for ^{13}C, using 1D spectra and 2D homo- and heteronuclear correlation experiments (^1H,^{13}C, DEPT, H,H-COSY, HSQC, and HMBC). The NMR data are listed in Tables 1 and 2. Negative-ion atmospheric pressure chemical ionization mass spectra (APCI-MS) were acquired at the Medical University of South Carolina, Regional Mass Spectroscopy Center using a Finnigan MAT LCQ. High-resolution (HR-APCI) mass spectra were acquired at the University of South Carolina, Department of Biochemistry and Chemistry facilities in Columbia, SC, using a VG70SQ double-focusing magnetic sector mass spectrometer. Retention times (t_R) were determined on a Waters HPLC system (Delta 600, M32 add-on single system, with a photodiode array detector model 996) using a Symmetry C18 (4.6 mm × 250 mm, Waters) reversed phase column with acetonitrile and 0.1% trifluoroacetic acid in water as solvent. A linear gradient from 10 to 100% acetonitrile in 30 min, at a flow rate of 1 mL/min was used. Detection was monitored at 280 nm. R_f values of the isolated compounds were determined on silica with a solvent mixture of chloroform/methanol/acetic acid/water (58:7:3.5:1). The UV spectra were recorded on a Beckman DU 650 spectrophotometer and IR spectra were obtained from a pure sample on KCl disks in a Mattson Genesis II FT FT-IR spectrometer. CD spectra were recorded on a AVIV circular dichroism spectropolarimeter (model 60DS) in a 1 cm cell.

11: 9-*C*-olivosyl-4-*O*-demethylpremithramycinone, $C_{26}H_{26}O_{12}$; APCI-MS *m/z* (relative intensity) $[M]^-$ 530 (40), $[M\text{-}OH]^-$ 513 (100); HR-APCI *m/z* $[M]^-$ calcd 530.1424, found 530.1428; t_R = 18.05 min; R_f = 0.21; UV (MeOH) λ_{max} (ϵ): 426 (6800), 322 (6700), 285 (32100), 239 (20,800) nm; (MeOH−HCl) λ_{max} (ϵ): 417 (7900), 327 (5300), 281 (27900), 231 (27000) nm; (MeOH−NaOH) λ_{max} (ϵ): 417 (10700), 278 (30100), 228 (19400) nm; FT-IR (KCl) ν 3297, 2970, 2910, 2832, 2363, 2338, 1676, 1636, 1580, 1522, 1448, 1420, 1337, 1270, 1162, 1125 cm^{-1}; CD (MeOH) λ_{extr} (Θ_D^{20}): 218 (−11500), 224 (−12700), 236 (−10600), 264 (−26500), 382 sh (37800), 469 (48400) nm.

12: 9-*C*-olivosylpremithramycinone, $C_{27}H_{28}O_{12}$; APCI-MS *m/z* (relative intensity) $[M - H]^-$ 543 (100); HR-APCI was unable to be obtained for this compound; t_R = 22.5 min; R_f = 0.57; UV (MeOH) λ_{max} (ϵ): 421 (18500), 326 (10400), 279 (65800), 223 (49000) nm; (MeOH−HCl) λ_{max} (ϵ): 415 (15700), 326 (9200), 278 (52500), 227 (59200) nm; (MeOH−NaOH) λ_{max} (ϵ): 421 (21900), 280 (57500) nm; FT-IR (KCl,) ν 3376, 2929, 2830, 2323, 1676, 1633, 1590, 1508, 1444, 1339, 1271, 1158, 1121 cm^{-1}; CD (MeOH) λ_{extr} (Θ_D^{20}): 227 (18000), 269 (−7146), 300 (5000), 312 (1800), 372 (43500), 433 (38000), 483 (47800) nm.

13: 9-*C*-mycarosyl-4-*O*-demethylpremithramycinone, $C_{27}H_{28}O_{12}$; APCI-MS *m/z* (relative intensity) $[M]^-$ 544 (40), $[M - OH]^-$ 527 (100); HR-APCI *m/z* $[M]^-$ calcd 544.1581, found 544.1575; t_R = 19.7 min; R_f = 0.30; UV (MeOH) λ_{max} (ϵ): 428 (9800), 324 (6700), 284 (30600), 231 (22000) nm; (MeOH−HCl) λ_{max} (ϵ): 417 (7400), 326 (5100), 281 (26700), 232 (27700) nm; (MeOH−NaOH) λ_{max} (ϵ): 417 (10100), 277 (28800) nm; FT-IR (KCl) ν 3332, 2973, 2930, 2359, 2332, 1684, 1635, 1590, 1525, 1448, 1427, 1373, 1333, 1275, 1165, 1125 cm^{-1}; CD (MeOH) λ_{extr} (Θ_D^{20}): 216 (−1400), 266 (−21800), 320 sh (2300), 391 sh (43000), 473 (53000) nm.

14: 9-*C*-mycarosylpremithramycinone, $C_{28}H_{30}O_{12}$; APCI-MS *m/z* (relative intensity) $[M]^-$ 558 (95), $[M - OH]^-$ 541 (60), $[M - OH - CH_3 - COCH_3]^-$ 483 (100); HR-APCI *m/z* $[M]^-$ calcd 558.1737, found 558.1750; t_R = 23.7 min; R_f = 0.57; UV (MeOH) λ_{max} (ϵ): 425 (11400), 324 (5500), 280 (38200), 239 (24500) nm; (MeOH−HCl) λ_{max} (ϵ): 419 (9800), 278 (6000), 280 (32000) 231 (33500) nm; (MeOH−NaOH) λ_{max} (ϵ): 417 (12900), 280 (36600) nm; FT-IR (KCl) ν 3332, 2970, 2932, 2835, 2370, 2334, 1675, 1636, 1590, 1517, 1448, 1424, 1372, 1330, 1272, 1162, 1124 cm^{-1}; CD (MeOH) λ_{extr} (Θ_D^{20}): 220 (−3400), 225 (−6900), 242 (−900), 269 (−18000), 281 (2000), 291 (−36400), 318 sh (−5100), 378 (40700), 423 (32600), 485 (51000) nm.

15: 9-*C*-diolivosylpremithramycinone, $C_{33}H_{38}O_{15}$; APCI-MS *m/z* (relative intensity) $[M]^-$ 674 (100), $[M - OH]^-$ 657 (60), $[M - OH - CH_3 - COCH_3]^-$ 599 (85); HR-APCI *m/z* $[M]^-$ calcd 674.2211, found 674.2195; t_R = 21.5 min; R_f = 0.53; UV (MeOH) λ_{max} (ϵ): 424 (11300), 326 (5800) 281 (38100), 241 (23800) nm; (MeOH−HCl) λ_{max} (ϵ): 418 (9400), 326 (5800), 280 (31900), 231 (31900) nm; (MeOH−NaOH) λ_{max} (ϵ): 419 (12500), 279 (35600) nm; FT-IR (KCl) ν 3337, 2970, 2929, 2370, 2341, 1683, 1634, 1577, 1521, 1448, 1424, 1373, 1334, 1291, 1163, 1124 cm^{-1}; CD (MeOH) λ_{extr} (Θ_D^{20}): 228 (9400), 262 (−4700), 300 sh (8800), 381 sh (58700), 470 (69500) nm.

Acknowledgment. This work was supported by a grant of the European Union (QLRT-1999-00095) to A.B. and J.A.S. as well as by grants from the U.S. Department of Defense and the National Institutes of Health (R01CA91901) to J.R. Dr. Daniel W. Bearden (Center for Coastal Environmental Health and Biomolecular Research, NOAA, Charleston, SC) is acknowledged for recording the NMR data on diolivosylpremithramycinone.

JA017385L

(21) Hopwood, D. A.; Bibb, M. J.; Chater, K. F.; Kieser, T.; Bruton, C. J.; Kieser, H. M.; Lydiate, D. J.; Smith, C. P.; Ward, J. M.; Schrempf, H. *Genetic Manipulation of Streptomyces. A Laboratory Manual*; The John Innes Foundation: Norwich. England, 1985.

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Chemistry & Biology 8 (2001) 1239–1252

Chemistry &Biology

www.elsevier.com/locate/chembiol

Research Paper

Elucidation of the function of two glycosyltransferase genes (*lanGT1* and *lanGT4*) involved in landomycin biosynthesis and generation of new oligosaccharide antibiotics

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Received 25 June 2001; revisions requested 29 August 2001; revisions received 26 September 2001; accepted 28 September 2001
First published online 7 November 2001

Abstract

Background: The genetic engineering of antibiotic-producing *Streptomyces* strains is an approach that became a successful methodology in developing new natural polyketide derivatives. Glycosyltransferases are important biosynthetic enzymes that link sugar moieties to aglycones, which often derive from polyketides. Biological activity is frequently generated along with this process. Here we report the use of glycosyltransferase genes isolated from the landomycin biosynthetic gene cluster to create hybrid landomycin/urdamycin oligosaccharide antibiotics.

Results: Production of several novel urdamycin derivatives by a mutant of *Streptomyces fradiae* Tü2717 has been achieved in a combinatorial biosynthetic approach using glycosyltransferase genes from the landomycin producer *Streptomyces cyanogenus* S136. For the generation of gene cassettes useful for combinatorial biosynthesis experiments new vectors named pMUNI, pMUNII and pMUNIII were constructed. These vectors facilitate the construction of gene combinations taking advantage of the compatible *Mun*I and *Eco*RI restriction sites.

Conclusions: The high-yielding production of novel oligosaccharide antibiotics using glycosyltransferase gene cassettes generated in a very convenient way proves that glycosyltransferases can be flexible towards the alcohol substrate. In addition, our results indicate that LanGT1 from *S. cyanogenus* S136 is a D-olivosyltransferase, whereas LanGT4 is a L-rhodinosyltransferase.

Keywords: Urdamycin; Landomycin; Glycosyltransferase; Combinatorial biosynthesis; Vector system

1. Introduction

Many biologically active natural compounds are produced by actinomycetes, most of them by *Streptomyces* species. Since the first report on using genetic engineering techniques to create hybrid antibiotics the potential of manipulating biosynthetic pathways in *Streptomyces* has received much attention [1,2]. Many naturally occurring bioactive metabolites possess unusual carbohydrate moieties, which serve as molecular recognition elements important for biological activities. Without these sugar elements, the biological activities are often either completely abolished or dramatically decreased [3,4].

There is an increasing interest in antibiotics containing oligosaccharide structures. As oligosaccharide antibiotics are difficult to produce by chemical synthesis, the genetic engineering of oligosaccharide structures could be an important and very promising technique. Recently it has been shown that glycosyltransferases, responsible for the formation of oligosaccharide structures, may have a certain degree of flexibility towards nucleotide sugar donor but also towards the acceptor molecule [5–7].

Landomycin A, produced by *Streptomyces cyanogenus* S136, and urdamycin A, produced by *Streptomyces fradiae*

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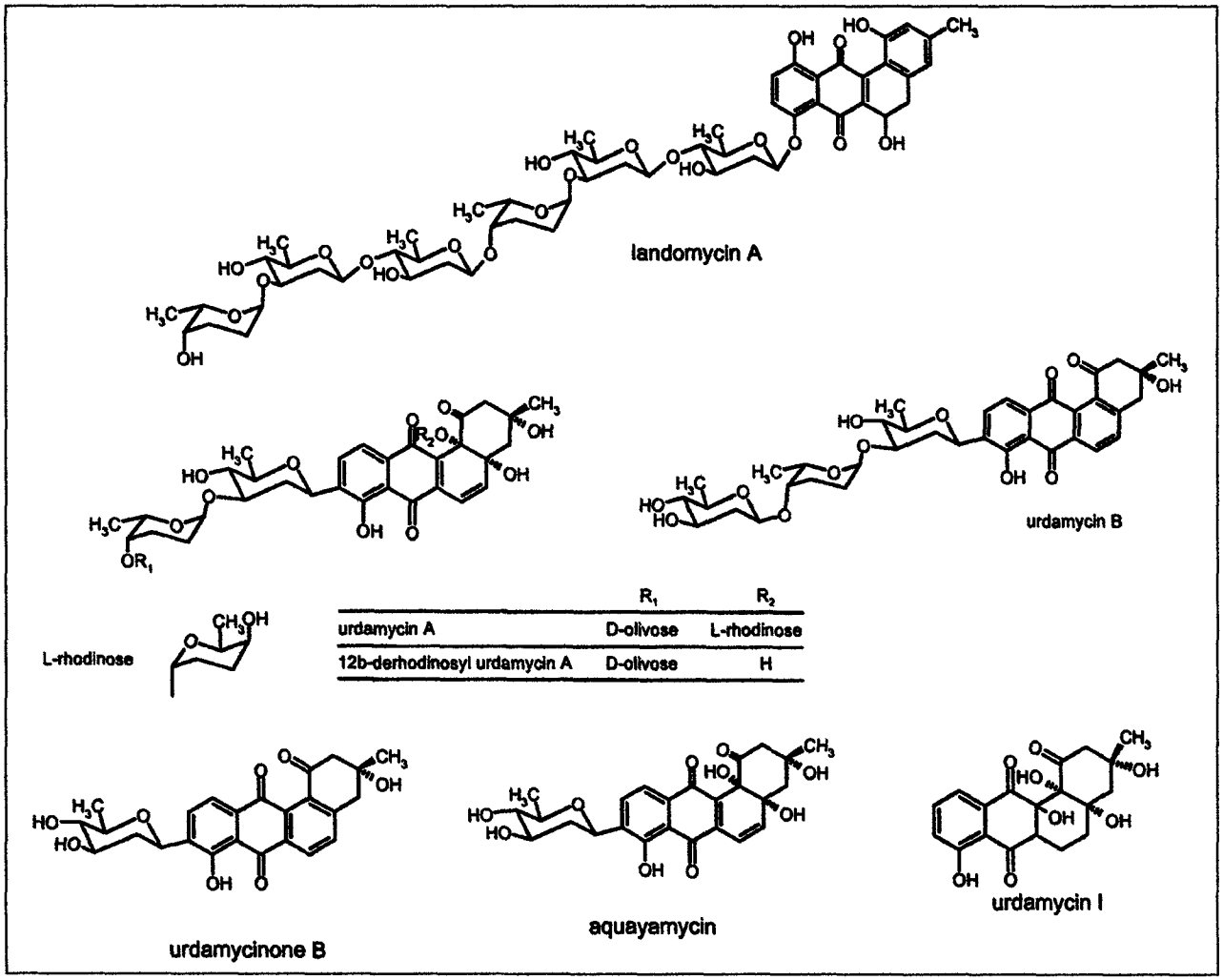
1074-5521/01/$ – see front matter
PII: S1074-5521(01)00091-6

Tü2717, belong to the angucycline group of antibiotics [8]. In various biological tests landomycin A showed interesting antitumor activities, in particular against prostata cancer cell lines [9], and it was shown that landomycin A inhibits DNA synthesis and G_1/S cell cycle progression [10]. In contrast, urdamycin A possesses only weak antitumor activity. One important difference between both compounds is the oligosaccharide side chain attached to the polyketide moiety. The hexasaccharide side chain of landomycin A is comprising two repeating trisaccharides each consisting of the sequence β-D-olivose-(4→1)-β-D-olivose-(3→1)-α-L-rhodinose (Fig. 1) [11]. The same deoxysugar building blocks are assembled to a trisaccharide chain in urdamycin A forming the sequence β-D-olivose-(3→1)-α-L-rhodinose-(4→1)-β-D-olivose [12]. This trisaccharide is connected to the aglycone by an unusual C-glycosidic bond and urdamycin A contains an additional L-rhodinose attached O-glycosidically at the 12b-position (Fig. 1).

The biosynthetic gene clusters of both compounds have been cloned and sequenced [6,13–16]. Four glycosyltransferase genes have been detected in each cluster. The function of glycosyltransferases involved in urdamycin biosynthesis has been elucidated [15,16] and the generation of a strain of *S. fradiae* Tü2717 with mutations in three of four glycosyltransferase genes producing monoglycosylated intermediates as aquayamycin has been described [16]. Here we report the successful expression of glycosyltransferase genes of the landomycin producer in a mutant of the urdamycin producer *S. fradiae* Tü2717, which led to the formation of various, new urdamycin derivatives with new saccharide side chains. We also introduce a novel cloning vector allowing the simple and convenient set up of gene cassettes.



	R_1	R_2
urdamycin A	D-olivose	L-rhodinose
12b-derhodinosyl urdamycin A	D-olivose	H

Fig. 1. Structures of landomycin A produced by *S. cyanogenus* S136 and urdamycin derivatives produced by *S. fradiae* and mutants *S. fradiae* A-x and *S. fradiae* A-0. For the urdamycins only A- and B-type aglycones are shown. These are produced as a complex with C- and D-type urdamycins without changes in the sugar moieties [21].

2. Results

2.1. Sequence comparison of glycosyltransferases from the landomycin and urdamycin biosynthetic gene cluster

It has been described that the comparison of the deduced products of all eight putative glycosyltransferase genes from the landomycin and urdamycin biosynthetic gene cluster with proteins in databases clearly implemented that they are involved in glycosylation steps [13,15,16]. Since the functions of the glycosyltransferases UrdGT1a (12b-L-rhodinosyltransferase), UrdGT1b (4A-D-olivosyltransferase), UrdGT1c (3'-L-rhodinosyltransferase) and UrdGT2 (9-D-olivosyltransferase) were determined unambiguously [15,16], we expected to predict the function of the glycosyltransferases LanGT1, LanGT2, LanGT3 and LanGT4 by sequence comparison. As shown in Fig. 2 the results were: (i) UrdGT2 resembles LanGT2, (ii) UrdGT1b and UrdGT1c are closely related to LanGT1 and LanGT3, and (iii) UrdGT1a shows the closest resemblance to LanGT4.

2.2. Generation of a S. fradiae mutant (mutant S. fradiae A-0) lacking all four glycosyltransferase genes of the urdamycin biosynthetic gene cluster

S. fradiae BF-1-1 [15] carrying an in-frame deletion in *urdGT2* was used to generate a mutant lacking all four glycosyltransferase genes from the urdamycin biosynthetic gene cluster. Mutant BF-1-1 was transformed with pKC-12-B2 [16], which has been constructed for the deletion of *urdGT1a*, *urdGT1b* and *urdGT1c*. Apramycin resistant colonies were obtained and single cross-over events were confirmed by hybridization using a ColE1 fragment from pKC1132 as probe. Integrants were grown without apramycin selection to allow for second cross-over. Apramycin sensitive clones were screened by PCR for the deletion of a 4 kb *Bam*HI–*Pst*I fragment which removes *urdGT1a*, *urdGT1b*, *urdInt* and *urdGT1c* from the chromosome. The deletion was confirmed by Southern hybridization. Chromosomal DNA from this mutant named *S. fradiae* A-0 and DNA from mutant BF-1-1 were isolated and digested with *Pst*I. Hybridization was carried out using a 5.2 kb *Sac*I fragment as a probe. Strain BF-1-1 showed the expected bands at 5.2, 2.6 and 2.4 kb whereas mutant A-0 showed one single band at 2.4 kb confirming the deletion (Fig. 3C). Secondary metabolites accumulated by mutant A-0 were urdamycin I (Fig. 1), urdamycin J (major components) and minor amounts of rabelomycin. The genotypes of all strains used in this study are shown in Fig. 3.

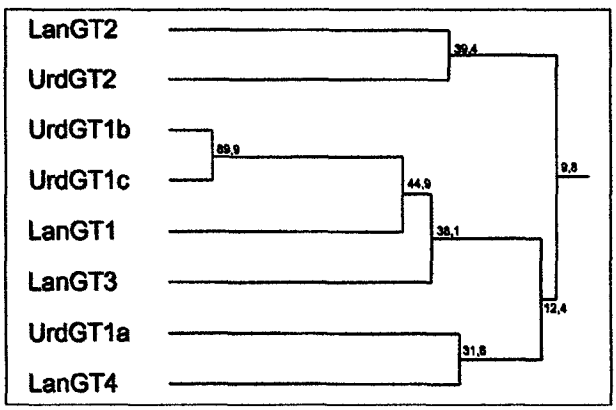


Fig. 2. Dendrogram derived from the comparison of urdamycin and landomycin glycosyltransferase sequences (accession numbers: UrdGT1a AAF00214, UrdGT1b AAF00215, UrdGT1c AAF00217, UrdGT2 AAF00209, LanGT1 AAD13555, LanGT2 AAD13553, LanGT3 AAD13559, LanGT4 AAD13562). The dendrogram was drawn based on an alignment built with DNASIS using the resettings of the program. Genetic similarity is given at points of branching.

Table 1
Important restriction sites located in the polylinker of pMUN vectors

Plasmid	Restriction sites
pMUN1	*Hin*dIII, *Mun*I, *Eco*RV, *Eco*RI, *Nsi*I, *Bam*HI, *Spe*I, *Xba*I
pMUN2	*Hin*dIII, *Mun*I, *Nco*I, *Nsi*I, *Nde*I, *Bgl*II, *Eco*RI, *Spe*I, *Xba*I
pMUN3	*Hin*dIII, *Mun*I, *Nco*I, *Nsi*I, *Nde*I, *Bgl*II, *Eco*RI, *Bam*HI, *Spe*I, *Xba*I

2.3. Construction of a novel vector system allowing a simple set up of gene cassettes

To facilitate the set up of gene cassettes for combinatorial biosynthesis several plasmids named pMUNI, pMUNII and pMUNIII were constructed. These vectors contain a single *Mun*I and a single *Eco*RI restriction site within the polylinker. Genes can be cloned into these vectors between both restriction sites. As *Mun*I and *Eco*RI are compatible, gene cassettes can be generated as depicted in Fig. 4. Important restriction sites of vectors pMUNI, pMUNII and pMUNIII are given in Table 1. pMUNI still retains the intact *lacZ'* from pBluescript allowing blue/white screening. Gene cassettes generated in one of these vectors can be restricted by *Hin*dIII and *Xba*I and can be ligated into the *Hin*dIII and *Xba*I sites of plasmid pUWL201 [17]. This plasmid contains the minimal replicon from pIJ101 [18] and the *tsr* gene from *Streptomyces azureus* conferring resistance to thiostrepton for propagation and selection in *Streptomyces*. The ColE1 replicon and the β-lactamase from pBluescript SK– are used for maintenance in *Escherichia coli*. The strong constitutive *ermE* promoter [19] allows the expression of genes in *Streptomyces* under control of the constitutive erythromycin resistance promoter.

2.4. Expression of single glycosyltransferase genes in S. fradiae A-x and S. fradiae A-0

As listed in Table 2 all genes were expressed singly in *S. fradiae* A-x and *S. fradiae* A-0. *urdGT2*, *lanGT1*, *lanGT2*, *lanGT3* and *lanGT4* were amplified by PCR, first cloned

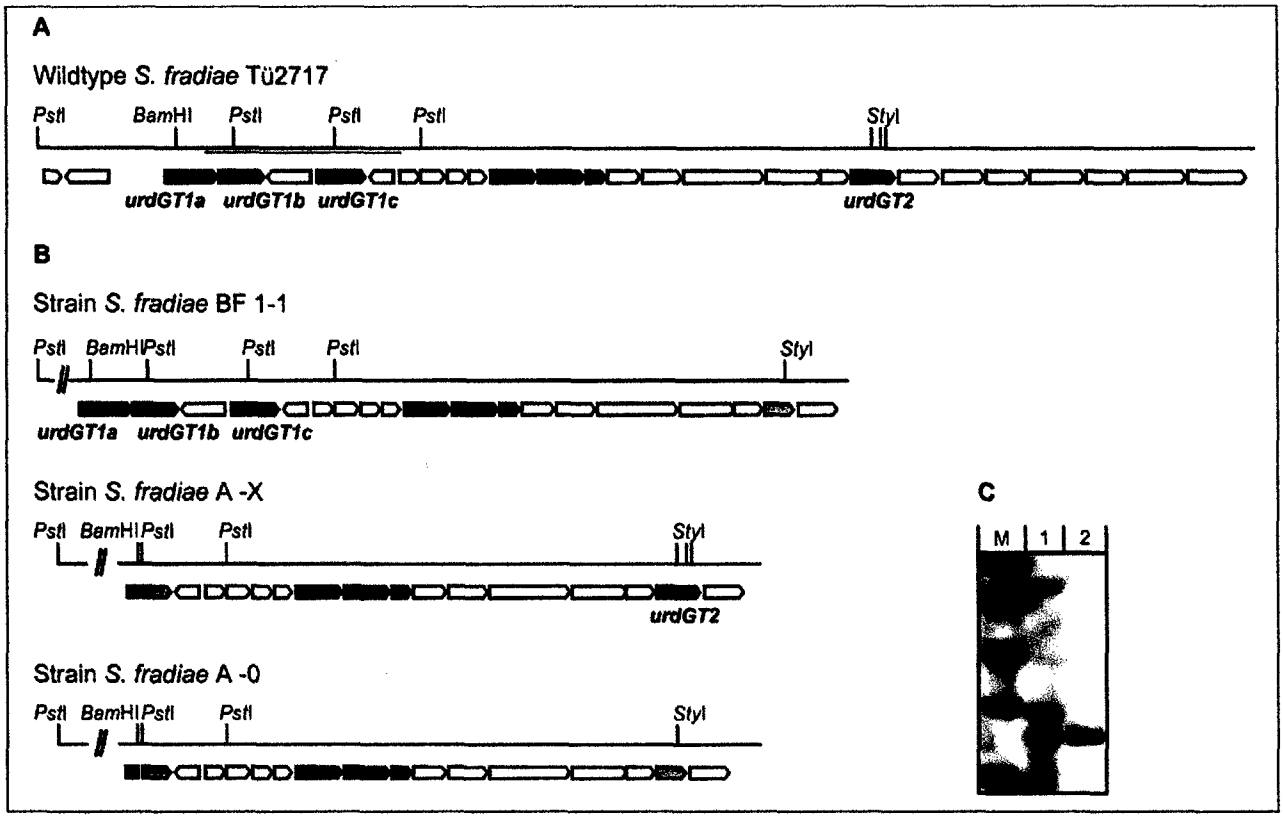


Fig. 3. A: Organization of the urdamycin biosynthetic gene cluster from *S. fradiae* Tü2717. Genes and their direction of transcription are indicated by arrows; active glycosyltransferase genes are shown in red, polyketide synthase genes in blue; the blue bar shows the localization of the fragment used as probe for hybridization; only relevant restriction sites are shown. B: Genotypes of glycosyltransferase gene mutant strains used in this study; inactive glycosyltransferase genes are shown in dark gray. C: Southern hybridization confirming the deletion in *S. fradiae* A-0; lane M: DIG-labelled molecular size marker with the following fragments shown: 1953 bp, 2799 bp, 3639 bp, 4899 bp, 6106 bp; lane 1: chromosomal DNA isolated from *S. fradiae* BF-1-1, digested with *PstI*; lane 2: chromosomal DNA isolated from *S. fradiae* A-0, digested with *PstI* (for details see text).

into the vector pMUNII and then transferred to the expression vector pUWL201. These constructs as well as constructs containing *urdGT1a*, *urdGT1b* and *urdGT1c* [16] were used to transform the A-x and A-0 mutants. The expected production of compounds 100-1 and 12b-derhodinosyl-urdamycin G (Fig. 5) was observed when *urdGT1c* was expressed in mutant A-x and compound 100-2 (Fig. 5) was found when *urdGT1a* was expressed. Also as expected, urdamycinone B and aquayamycin (Fig. 1) were produced when *urdGT2* was expressed in mutant A-0.

Thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) analysis of culture supernatants of mutant A-0 containing either *lanGT1*, or *lanGT2*, or *lanGT3*, or *lanGT4* did not show the formation of any new compound. However, new compounds were produced by mutant A-x containing either *lanGT1* or *lanGT4*. 12b-Derhodinosyl-urdamycin G (and various follow-up products including the previously not described photochemical rearrangement product, named lourdamycin), compound 100-1, and its new 5-hydroxy analog (Fig. 5) were the principal products of mutant A-x after expression of *lanGT4*. By experimental incidents we also isolated one plasmid containing two copies of *lanGT4*. Expression of this plasmid, which is a strong overexpression of the *lanGT4* gene, in mutant A-x resulted in 12b-derhodinosyl-urdamycin G plus large amounts (30% of total production) of urdamycin N and traces of the new urdamycin Q (Fig. 5). Small amounts of novel compounds, presumably ladamycins A_2 and B_2, were detected when extracts of *S. fradiae* A-x containing *lanGT1* were carefully analyzed by HPLC-mass spectrometry (MS).

2.5. *Coexpression of urdGT2, urdGT1a, urdGT1b and urdGT1c in S. fradiae A-0*

The successful implementation of the pMUN vectors requires all genes to be efficiently transcribed from the erythromycin resistance promoter. As a control, plasmids pUWL-u1a-u2 containing *urdGT1a* and *urdGT2* and pUWL-u2-u1a-u1b-u1c containing all four urdamycin glycosyltransferase genes were constructed and expressed in *S. fradiae* A-0. Compound 100-2 was identified when *urdGT1a* and *urdGT2* were expressed and urdamycin A production was restored to similar levels as in the wild-type after coexpression of all four genes (Table 2).

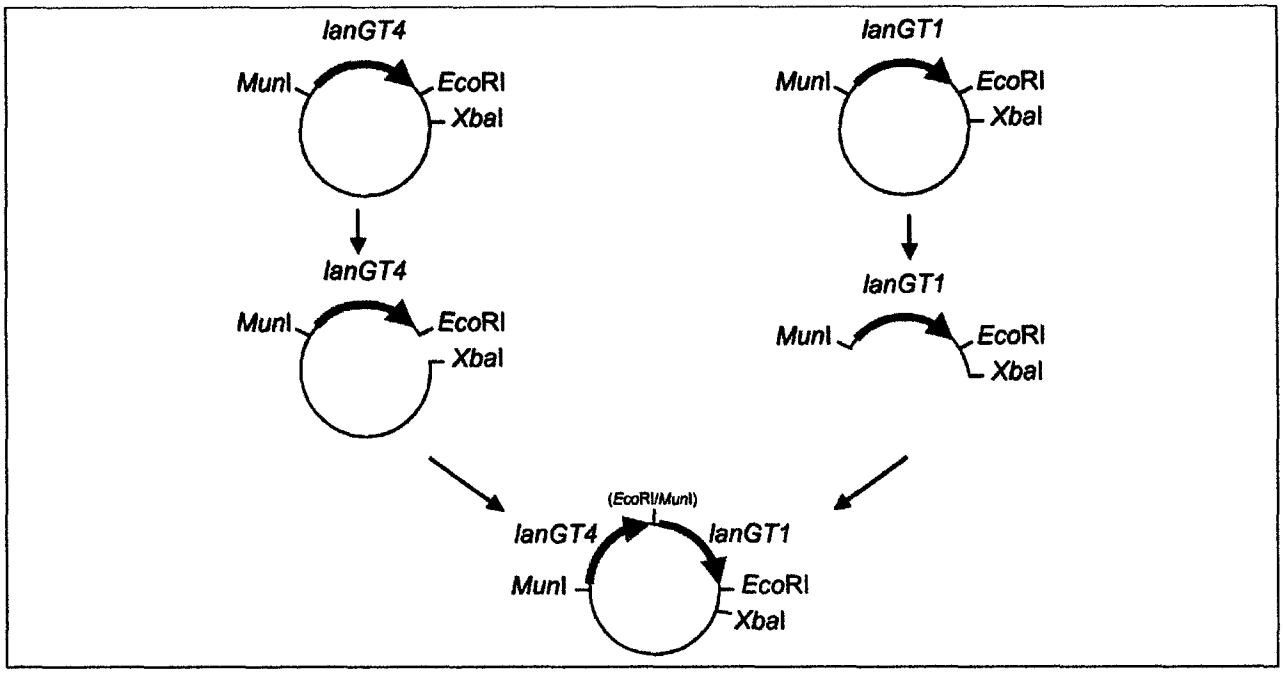


Fig. 4. Generation of a gene cassette containing *lanGT1* and *lanGT4* using the pMUN vector system.

2.6. *Coexpression of landomycin glycosyltransferase genes in S. fradiae A-x*

The expression of *lanGT1* and/or *lanGT4* in *S. fradiae* A-x resulted in the accumulation of hybrid antibiotics (see above). Gene cassettes containing *lanGT1*+either *lanGT2*, or *lanGT3* or *lanGT4* and *lanGT4*+either *lanGT2* or *lanGT3*, respectively, were constructed and expressed in mutant A-x. Analysis of supernatant of the strain containing *lanGT1*+*lanGT4* showed production of several novel compounds, namely ladamycin A_3, its follow-up product ladamycin D_3 and ladamycin B_3 (Fig. 5) at high levels (90% of total production). These compounds were also produced by mutants containing *lanGT1*+*lanGT4*+*lanGT2*, *lanGT1*+*lanGT4*+*lanGT3*, and the one which contained all four landomycin glycosyltransferase genes.

Table 2
Mutants, glycosyltransferase gene constructs and resulting metabolites

Strain	Plasmid	Expressed glycosyltransferase	Products of the recombinant strain
Wild-type	–		urdamycin A, urdamycin B, 12b-derhodinosyl-urdamycin A
A-0	pUWL201	–	urdamycin I, urdamycin J, rabelomycin
A-0	pUWL-urdGT2	UrdGT2	aquayamycin, urdamycinone B
A-0	pUWL-u1a-u2	UrdGT1a+UrdGT2	100-2, aquayamycin, urdamycinone B
A-0	pUWL-u2-u1b-u1c-u1a	UrdGT2+UrdGT1a+UrdGT1b+UrdGT1c	urdamycin A, 12b-derhodinosyl-urdamycin A, urdamycin G, 100-2, urdamycin B, 100-1
A-x	pUWL201	–	aquayamycin, urdamycinone B
A-x	pUWL-I1	LanGt1	aquayamycin, urdamycinone B, ladamycin A_2, ladamycin B_2
	pUWL-I1-I2	LanGT1+LanGT2	
	pUWL-I1-I3	LanGt1+LanGT3	
	pUWL-I1-I2-I3	LanGT1+LanGT2+LanGT3	
A-x	pUWL-I4	LanGT4	12b-derhodinosyl-urdamycin G, aquayamycin, 100-1, urdamycinone B
	pUWL-I2-I4	LanGT2+LanGT4	
	pUWL-I3-I4	LanGT3+LanGT4	
	pUWL-I2-I3-I4	LanGT2+LanGT3+LanGT4	
A-x	pUWL-I4-I4	LanGT4+LanGT4	12b-derhodinosyl-urdamycin G, 100-1, urdamycin N
A-x	pUWL-I4-I1	LanGT4+LanGT1	ladamycin A_3, ladamycin B_3
	pUWL-I1-I2-I4	LanGT1+LanGT2+LanGT4	
	pUWL-I1-I3-I4	LanGT1+LanGT3+LanGT4	
	pUWL-I1-I2-I3-I4	LanGT1+LanGT2+LanGT3+LanGT4	

Expression of *urdGT1a*, *urdGT1c* and gene cassettes containing various combinations of these genes in mutant A-x and the resulting metabolites have been described [17]. Expression of single glycosyltransferase genes and gene cassettes of the landomycin cluster in mutant A-0 and expression of *lanGT2*, *lanGT3* and *lanGT2*+*lanGT3* in mutant A-0 did not result in the production of new metabolites.

2.7. Structures

All structures, except those for ladamycins A_2 and A_3 (Fig. 5), were determined using nuclear magnetic resonance (NMR) and MS. Ladamycins A_2 and A_3 were identified through HPLC-MS. In both cases, the HPLC-UV revealed the same 5-hydroxy-1,4-naphthoquinone chromophore known from aquayamycin and urdamycin A, but a different retention time than all known urdamycins. Compared to aquayamycin, the mass of 616 g/mol for ladamycin A_2 (in agreement with $C_{31}H_{36}O_{13}$) indicates an additional olivose moiety, and the mass of 730 g/mol found for ladamycin A_3 indicates an additional olivose plus an additional rhodinose moiety. Ladamycin A_2 was observed in *S. fradiae* A-x containing *lanGT1* and ladamycin A_3 was observed in *S. fradiae* A-x after coexpressing *lanGT1* and *lanGT4*.

The existence of ladamycin A_3 is also confirmed indirectly through the structure of ladamycin D_3, its urdamycin D-type analog, found in *S. fradiae* A-x (*lanGT1*, *lanGT2*, *lanGT3*, *lanGT4*). The non-enzymatic conversion of urdamycin A-type into urdamycin D-type molecules through addition of transaminated tryptophan is well known [20]. The negative fast atom bombardment (FAB) mass spectrum confirms the molecular formula $C_{47}H_{51}NO_{16}$ (885; *m/z* found 885, 100%, M^-) of ladamycin D_3. The 1,4-linkage of the two olivose moieties in ladamycin D_3 follows from the $^3J_{C-H}$ couplings between 1A-H and C-4′ and between 4′-H and C-1A, both observed in the HMBC spectrum (Table 7) as well as from

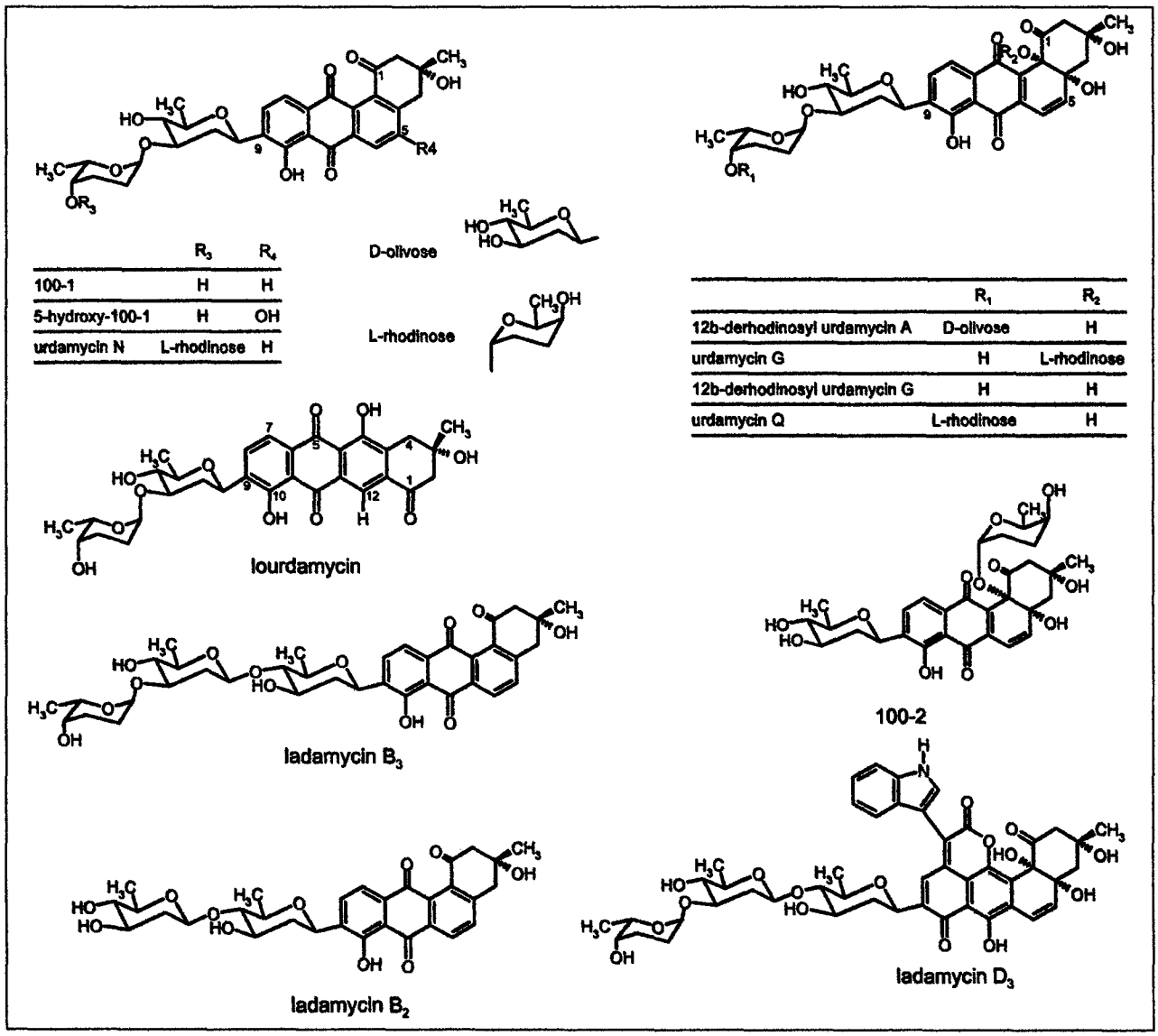


Fig. 5. Compounds produced by expression of glycosyltransferase genes in mutants *S. fradiae* A-x and *S. fradiae* A-0.

Table 3
Primers used for amplification of glycosyltransferase genes

Amplified gene	Name	Sequence
urdGT2	urdGT2F	TGGGCCGAGGCAATTGCATCCGGCACTC
	urdGT2R	GGTGCCGGATCCGCCCGCCAGCACAAG
lanGT1	lanGT1F	TCGGCACAATTGGCAGGAGACGCATATG
	lanGT1R	CGACTCGTGAGATCTCGCGGTCGGTCA
lanGT2	lanGT2F	CACCTCAAGCTTCAATTGCAGCAGGGAGTATCCGTG
	lanGT2R	CGATGCGAGATATCAGACCAG
lanGT3	lanGT3F	GCGTGCCAATTGCCAAGGCATATGATG
	lanGT3R	GGACCGGCCGAGATCTGACGAGGGG
lanGT4	lanGT4F	GAATCCCAATTGGAGAACCATATGCGTG
	lanGT4R	TCCTGACGGGCGGAGATCTTCGGCGGC

Restriction sites introduced for cloning are underlined.

the ^{13}C downfield shift observed for C-4′ (δ 88.2, see Table 6).

Ladamycin B_2 was discovered in *S. fradiae* A-x (*lanGT1*). Its structure is suggested from significant signals, observed in the ^1H NMR spectrum, which reveals two aromatic A–B systems as well as two olivose moieties.

Ladamycin B_3, found in *S. fradiae* A-x following coexpression of *lanGT1* and *lanGT4*, is a structural isomer of urdamycin B [21], as the high-resolved negative FAB-MS reveals ($C_{37}H_{44}O_{13}$, HR calculated 696.2782, found 696.2802). It differs from urdamycin B in the sequence of its saccharide chain, which is α-L-rhodinose-(1→3)-β-D-olivose-(1→4)-β-D-olivose. This saccharide chain sequence follows from the observed long-range couplings (HMBC, Table 7) and the chemical shifts of the carbon signals (δ 88.6 for C-4′, δ 77.3 for C-3A). The $^3J_{C-H}$ couplings between 4′-H and C-1A and between 1A-H and C-4′ prove the 1,4-linkage of the two olivose units, whereas the coupling between 1B-H and C-3A proves the linkage of the terminal rhodinose to 3-OH of olivose A.

The major product of *S. fradiae* A-x containing *lanGT4* is 12b-derhodinosyl-urdamycin G. Its MS data (negative atmospheric pressure chemical ionization (APCI)-MS, *m/z* 600.2, 8%, M^-, *m/z* 582.3, 100%, $M-H_2O$) confirm its molecular formula of $C_{31}H_{36}O_{12}$ (calculated 600.2207). This indicates an additional rhodinose compared to aquayamycin. The NMR data (Table 5 and 6) confirm the presence of a rhodinose moiety, and also its linkage to the 3′-OH group of the C-glycosidically bound D-olivose moiety (^{13}C NMR and HMBC data, Tables 6 and 7). Distinct signals of the rhodinose (sugar A) are for instance the anomeric proton and carbon (δ_H 4.96; δ_C 95.2), which appear in the ^1H NMR spectrum as a broad singlet due to its α-glycosidic linkage and in the anomeric region below δ 100 in the ^{13}C NMR spectrum. Another distinct signal of the rhodinose is the broad quartet (δ 4.18, Table 5) of 5A-H. The linkage of this rhodinose to the 3′-position (of the C-glycosidically bound D-olivose) is indicated by the typical downfield shift of this signal in the ^{13}C NMR (δ 78.7, Table 6) and is proven unambiguously by the $^3J_{C-H}$ couplings between 1A-H and C-3′ as well as between 3′-H and C-1A (both were observed in the HMBC spectrum, Table 7).

The structure of the new compound 5-hydroxy-100-1, which was also discovered in *S. fradiae* A-x containing *lanGT4*, was deduced from its high-resolved negative FAB mass spectrum (the $M-H^-$ was observed, calculated for $C_{31}H_{33}O_{11}$ 581.2023, found 581.2027) indicating one oxygen atom more than for the known compound 100-1 [22], and the NMR data (Tables 5–7). The position of the additional OH group in 5-hydroxy-100-1 follows from the 6-H signal, which appears as singlet (δ 7.78) in contrast to 100-1, where this is a doublet of an AB system [22]. That the OH group is located at 5- and not at 6-position could be unambiguously clarified through the HMBC spectrum, in which long-range C–H couplings were observed between this singlet and C-4a, C-5, C-6a, C-7 and C-12.

Another new compound from the same strain, which has never been observed previously, is lourdamycin, which

Table 4
Oligonucleotides used for construction of pMUN vectors

Vector	Oligosequences	Ligated to	Cut with
pMUNI	AGCTTCAATTGGATATCGAATTCATGCATG CTAGCATGCATGAATTCGATATCCAATTGA	pBluescript	*Hin*dIII/*Bam*HI
pMUNII	AATTGCCATGGATGCATATGAGATCTGAATTC GATCGAATTCAGATCTCATATGCATCCATGGC	pMUNI	*Mun*I/*Bam*HI
pMUNIII	AATTGCCATGGATGCATATGAGATCTGAATTCG GATCCGAATTCAGATCTCATATGCATCCATGGC	pMUNI	*Mun*I/*Bam*HI

Overhanging compatible ends used for insertion of the oligonucleotide are underlined.

Table 5
^{1}H NMR data of ladamycin B_3, ladamycin D_3, 5-hydroxy-100-1, 12b-derhodinosyl-urdamycin G and lourdamycin in d_6-acetone at 400 MHz

Proton	Ladamycin B_3	Ladamycin D_3	5-Hydroxy-100-1	12b-Derhodinosyl-urdamycin G	Lourdamycin
2-H_{eq}	2.87 dd (14, 1.5)[a]	2.81 dd (13, 3)[a]	2.82 dd (13.5, 1.5)	2.67 dd (12.5, 3)	2.82 dd (16, 2)[a]
2-H_{ax}	3.08 d (14)[b]	3.28 d (13)	3.05 d (13.5)	2.97 d (12.5)	2.94 d (16)
3-CH_3	1.47 s	1.21 s	1.48 s	1.22 s	1.52 s
4-H_{eq}	3.20 dd (17, 1.5)	1.94–2.12[b]	3.25 dd (18, 1.5)	ca. 2.06[b]	3.36 dd (18, 2)
4-H_{ax}	3.31 d (17)	1.94–2.12[b]	2.99 d (18)	2.23 d (15)	3.08 d (18)
5-H	7.73 d (8)	6.09 d (10)	–	6.44 d (10)	–
6-H	8.30 d (8)	7.00 d (10)	7.78 s	6.81 d (10)	–
7-H	–	–	–	–	7.91 d (8)
8-H	–	–	–	–	8.00 d (8)
10-H	7.92 d (8)	8.15 s	7.88 d (8)	7.88 d (8)	–
11-H	7.60 d (8)	–	7.56 d (8)	7.54 d (8)	–
12-H	–	–	–	–	8.36 s
5″-H	–	8.01 s	–	–	–
7″-H	–	7.58 d (7.5)[d]	–	–	–
8″-H	–	7.23 dd (7.5, 7)	–	–	–
9″-H	–	7.15 dd (7.5, 7)	–	–	–
10″-H	–	7.57 d (7.5)[d]	–	–	–
1′-H	4.90 dd (11, 2)	4.74 d (10)	4.87 dd (11, 1.5)	4.85 dd (11, 1.5)	4.92 dd (11.5, 2)
2′-H_{eq}	2.46 ddd (13, 5, 2)	2.44 ddd (13, 5, 1.5)	2.51 ddd (13, 5, 2)	2.49 ddd (13, 5, 2)	2.57 ddd (13, 5, 2)
2′-H_{ax}	1.30–1.38[b,c]	1.16–1.26[b,c]	1.26–1.43[b,c]	1.26–1.44[b,c]	1.26–1.43[b,c]
3′-H	3.74 ddd (11, 9, 5)	3.68 ddd (11, 9, 5)[b]	3.78 ddd (11, 9, 5)	3.77 ddd (11, 8.5,5)	3.81 ddd (11, 9, 5)
4′-H	3.14 dd (9, 9)	2.90 dd (9, 9)[a]	3.15 dd (9, 9)	3.14 dd (9, 9)	3.17 ddd (9, 9, 3)
5′-H	3.55 dq (9, 6)[b]	3.44 dq (9, 6)[b]	3.48 dq (9, 6)	3.48 dq (9, 6)	3.51 dq (9, 6)[b]
6′-CH_3	1.35 d (6)	1.05 d (6)	1.35 d (6)	1.35 d (6)	1.37 d (6)
1A-H	4.74 dd (10, 2)	4.61 dd (10, 1.5)	4.98 br s	4.96 br s	4.99 br s
2A-H_{eq}	2.41 ddd (13, 5, 2)	2.33 ddd (12.5, 5, 1.5)	1.26–1.43[b,c]	1.26–1.44[b,c]	1.26–1.43[b,c]
2A-H_{ax}	1.40–1.54[b,c]	1.39–1.45[b,c]	1.98–2.09[b,c]	2.02–2.09[b,c]	2.02–2.09[b,c]
3A-H_{eq}	–	–	1.98–2.09[b,c]	2.02–2.09[b,c]	2.02–2.09[b,c]
3A-H_{ax}	3.67 ddd (12, 9, 5)	3.61 ddd (11.5, 9, 5)[b]	1.58–1.64[c]	1.59–1.64[c]	1.59–1.64[c]
4A-H	3.09 ddd (9, 9, 2.5)[b]	3.05 ddd (9, 9, 3)	3.53 br s	3.54 br s	3.55 br s[b]
5A-H	3.43 dq (9, 6)	3.37 dq (9, 6)[b]	4.19 dq (6.5, 1)	4.18 dq (6.5, 1)	4.20 dq (6.5, 1)
6A-CH_3	1.30 d (6)	1.26 d (6)	1.14 d (6.5)	1.14 d (6.5)	1.15 d (6.5)
1B-H	4.95 br s	4.91 br s	–	–	–
2B-H_{eq}	1.40–1.54[b,c]	1.39–1.45[b,c]	–	–	–
2B-H_{ax}	2.00–2.10[b,c]	1.94–2.12[b,c]	–	–	–
3B-H_{eq}	2.00–2.10[b,c]	1.94–2.12[b,c]	–	–	–
3B-H_{ax}	1.59–1.66[c]	1.58–1.65[c]	–	–	–
4B-H	3.54 br s	3.53 br s	–	–	–
5B-H	4.17 dq (6.5, 1)	4.15 dq (6.5, 1)	–	–	–
6B-CH_3	1.12 d (6.5)	1.10 d (6.5)	–	–	–
OH signals[e]	4.66 d (2.5), 4.52, 4.17	5.25, 4.90, 4.72, 4.64 d (3), 4.45, 3.12	4.64	12.33, 5.24, 4.74, 4.63, 4.61	13.10, 13.12, 4.64 d (3), 4.14

δ in ppm, (J in Hz). Coupling partners confirmed by gCOSY experiments.
[a]Obscured by water, visible after addition of D_2O.
[b]Obscured by solvent or other signal(s).
[c]Complex.
[d]Assignments are interchangeable; br = broad signal.
[e]Not all OH signals were observed due to water in the samples.

has a linearly assembled tetracyclic aglycone. This could be deduced from the NMR data. A photochemical rearrangement of aquayamycin into the analogous linearly assembled tetracene-1,6,12-trione derivative has been described by Umezawa et al. [23]. The ^{1}H NMR data of the aglycone moiety of lourdamycin (that includes the C-glycosidically bound D-olivose) are in agreement with those described for the rearranged aquayamycin. Thus, it is likely that lourdamycin derives from 12b-derhodinosyl-urdamycin G, presumably because of the influence of light during the fermentation and/or purification process. The rearranged aglycone structure follows from the long-range C–H couplings observed in the HMBC spectrum, in particular the couplings between 12-H and C-1, C-4a, C-5a and C-11 (Table 7).

After expression of pUWL-l4-l4 in *S. fradiae* A-x the new urdamycin Q was found. Since only about 0.3 mg of the purified compound was available for characterization, only a ^{1}H NMR spectrum in d_6-acetone was obtained. This clearly shows the aromatic (δ 7.93 and 8.01) as well

Table 6
^{13}C NMR data (from HMBC and HSQC spectra) of ladamycin B_3, ladamycin D_3, 5-hydroxy-100-1, 12b-derhodinosyl-urdamycin G and lourdamycin in d_6-acetone (100.6 MHz)

Carbon	Ladamycin B_3	Ladamycin D_3	5-Hydroxy-100-1	12b-Derhodinosyl-urdamycin G	Lourdamycin
1	196.2 s	206.6 s	196.5 s	205.2 s	195.8 s
2	53.3 t	52.0 t	53.1 t	51.9 t	51.2 t
3	71.8 s	75.3 s	71.6 s	76.6 s	70.3 s
3-CH_3	29.5 q	29.4 q	29.8 q	29.5 q	29.4 q
4	43.9 t	43.9 t	37.8 t	43.6 t	36.6 t
4a	149.4 s	80.5 s	137.1 s	81.0 s	138.8 s
5	134.2 d	137.2 d	160.6 s	145.7 d	161.5 s
5a	–	–	–	–	117.3 s
6	128.8 d	118.3 d	112.4 d	116.8 d	188.4 s
6a	133.5 s	123.4 s	134.7 s	138.5 s	132.1 s
7	188.4 s	155.7 s	188.9 s	189.2 s	119.4 d
7a	115.1 s	n.o.	115.2 s	114.3 s	–
8	158.0 s	186.8 s	157.9 s	157.6 s	133.6 d
9	136.9 s	142.8 s	136.4 s	138.2 s	139.2 s
10	133.6 d	135.2 d	133.5 d	133.4 d	159.0 s
10a	–	–	–	–	115.8 s
11	118.7 d	n.o.	118.4 d	118.9 d	188.2 s
11a	134.5 s	116.5 s	134.6 s	131.0 s	n.o.
12	182.7 s	n.o.	181.2 s	182.3 s	115.7 d
12a	136.3 s	128.4 s	127.1 s	139.5 s	138.5 s
12b	136.4 s	78.1 s	139.0 s	76.9 s	–
2″	–	n.o.	–	–	–
3″	–	127.2 s	–	–	–
4″	–	108.2 s	–	–	–
5″	–	131.9 d	–	–	–
6a″	–	136.6 s	–	–	–
7″	–	112.3 d	–	–	–
8″	–	122.8 d	–	–	–
9″	–	121.1 d	–	–	–
10″	–	120.4 d	–	–	–
10a″	–	127.2 s	–	–	–
1′	71.0 d	70.7 d	71.0 d	71.0 d	71.1 d
2′	39.1 t	39.2 t	37.2 t	37.0 t	36.9 t
3′	70.6 d	70.4 d	78.9 d	78.7 d	78.7 d
4′	88.6 d	88.2 d	75.9 d	75.8 d	75.8 d
5′	74.6 d	74.3 d	76.3 d	76.4 d	76.3 d
6′	17.5 q	17.3 q	17.9 q	17.9 q	17.9 q
1A	101.0 d	100.8 d	95.3 d	95.2 d	95.3 d
2A	36.5 t	36.4 t	23.9 t	23.8 t	23.8 t
3A	77.3 d	76.6 d	25.6 t	25.5 t	25.5 t
4A	75.2 d	74.9 d	66.4 d	66.3 d	66.4 d
5A	72.3 d	72.2 d	67.1 d	67.2 d	67.1 d
6A	17.4 q	17.4 q	16.6 q	16.7 q	16.6 q
1B	95.4 d	95.1 d	–	–	–
2B	23.9 t	23.9 t	–	–	–
3B	25.4 t	25.5 t	–	–	–
4B	66.4 d	66.3 d	–	–	–
5B	67.2 d	67.1 d	–	–	–
6B	16.7 q	16.5 q	–	–	–

δ in ppm, ^{13}C multiplicities from gHSQC and gHMBC experiments. n.o. = not observed.

as the olefinic AB system (δ 6.42 and 6.81), characteristic for the aquayamycin aglycone, and signals for three sugars. The C-glycosidically bound D-olivose (e.g. δ 4.94, d, J = 10 Hz, 1′-H; δ 4.01, m, 3′-H), and the two rhodinose moieties are identified by typical signals (e.g. δ 5.0 s and 5.19 s, 1A-H and 1B-H; δ 4.23 q and 4.38 q, 5A-H and 5B-H) in comparison with the known urdamycin O [6]. The data also reveal that urdamycin Q lacks the L-rhodinose at C-12-O, which occurs in urdamycin O, and which can easily be identified through its typical ^1H signals [6].

The spectroscopic data (NMR, MS), R_f values and HPLC retention times of all known urdamycin derivatives, such as 100-1 (found in *S. fradiae* A-X after expression of *lanGT4*), and of urdamycin N (found in *S. fradiae* A-X with twice *lanGT4*) were identical with those previously described.

3. Discussion

Many antibiotics have their aglycone moieties assembled first followed by decoration with sugar moieties to produce the final bioactive compound. In recent years several glycosyltransferase genes have been isolated from antibiotic-producing organisms. Among these glycosyltransferase genes are *lanGT1*, *lanGT2*, *lanGT3* and *lanGT4* of the landomycin cluster [13], *urdGT1a*, *urdGT1b*, *urdGT1c* and *urdGT2* of the urdamycin cluster [15,16], those for erythromycin [24,25], megalomicin [26], mithramycin [27,28], elloramycin [29] and oleandomycin biosynthesis [30], *graORF14* of the granaticin cluster [31,32] and *dnrS*/*dnmS* of the daunorubicin biosynthetic pathway [33,34]. Functions of some of these genes have been determined mostly by gene inactivation experiments [15,16, 24,25,33] and in a few cases by heterologous expression [26,29,35]. Based on sequence comparison of the deduced amino acid sequences of the landomycin glycosyltransferase genes with the closely related urdamycin glycosyltransferase genes, whose functions were known, some initial hypothesis could be raised. We expected LanGT1 and LanGT3 to be involved in the elongation of the hexasaccharide chain, LanGT2 to be responsible for the attachment of a D-olivose to the polyketide moiety, and LanGT4 to be a rhodinosyltransferase. Recently, the knowledge on glycosyltransferase genes and other sugar biosynthetic genes has been exploited for the production of novel glycosylated hybrid antibiotics. Systems constructed by different groups rely on feeding of aglycones to a modified host, which harbors suitable glycosyltransferase and sugar biosynthetic genes [36,37]. Liu and coworkers produced several new derivatives of methymycin/pikromycin by deleting endogenous sugar biosynthetic genes [7,38] and coexpression of the *calH* gene from the calicheamine pathway [39]. Two recent examples describe the coexpression of sugar and aglycone biosynthetic genes to generate novel glycosylated anthracyclines or macrolides, respectively [40,41].

Some flexibility of glycosyltransferases especially towards their sugar co-substrates has been described making these glycosyltransferases attractive tools for combinatorial biosynthesis. Only a low number of pharmaceutically important drugs are containing two, three or even more deoxysugars. The chemical synthesis of such compounds is very difficult to proceed and therefore impractical, and nearly impossible to be established in large scale. With glycosyltransferase genes of the landomycin and urdamycin clusters in hand we wanted to explore whether landomycin/urdamycin hybrids carrying modified sugar side chains could be generated by expressing glycosyltransferase gene cassettes.

In addition to mutant *S. fradiae* A-x, in which three of four urdamycin glycosyltransferases had been deleted, we generated a second host (mutant *S. fradiae* A-0) carrying no functional glycosyltransferase gene. Since both mutants are still capable to produce dTDP-D-olivose and dTDP-L-rhodinose, which are likely to serve as co-substrates of the landomycin glycosyltransferases, both hosts were therefore suitable for the heterologous expression of these genes.

New cloning vectors (pMUNI, pMUNII and pMUNIII) were generated based on the compatibility of the restriction sites *Eco*RI and *Mun*I. A major advantage of these vectors is that once a number of biosynthetic genes cloned in either pMUNI, pMUNII or pMUNIII are available, they can be rapidly combined to generate large gene cassettes. Glycosyltransferase genes of the urdamycin cluster were used to test this gene cassette approach. *S. fradiae* A-0 containing a plasmid with *urdGT1a*, *urdGT1b*, *urdGT1c* and *urdGT2* was capable to produce urdamycin A in the same amount as the wild-type supporting the use of the pMUN vector strategy for combinatorial biosynthesis.

Based on this system we tested the possibility of producing novel oligosaccharide antibiotics by expressing landomycin glycosyltransferase genes in mutants A-x and A-0. None of the glycosyltransferases was accepting products of mutant A-0 as substrate, which indicates a high specificity of the glycosyltransferase responsible for the attachment of the first D-olivose to the aglycone. In contrast, aquayamycin and urdamycinone B, the main products of mutant A-x, were accepted as substrates by both LanGT1 and LanGT4. Expression of these genes, alone or in combination, resulted in the accumulation of 12b-derhodinosyl-urdamycin G and other novel compounds, such as urdamycin Q, ladamycin B_2, ladamycins A_3 and D_3. Expression of *lanGT3* and *lanGT2* or coexpression with *lanGT1* and/or *lanGT4* in our hosts did not lead to the production of new compounds with longer saccharide chains. Either these enzymes are more specific towards their alcohol substrates than LanGT1 or LanGT4 and we were not able to provide a suitable substrate yet, or the export system of *S. fradiae* limits the secretion of compounds containing more than three sugars in the 9-side chain.

The expression of *lanGT1* alone or together with *lanGT4* resulted in the formation of novel hybrid compounds. These compounds contain two 1,4-connected D-olivose units, as known from the landomycin hexasaccharide. Thus, these compounds combine structural elements of both landomycins and urdamycins and were therefore designated ladamycins. The lower production level of ladamycin A_2 compared to ladamycin A_3 might be attributed to a slow glycosylation rate of aquayamycin by LanGT1, while the second glycosylation step, the attachment of the L-rhodinose, occurs faster. Although ladamycins A_2 and A_3 were only recognized through HPLC-MS, sufficient NMR data exist for ladamycins B_3 and D_3, the latter being a follow-up product of ladamycin A_3. These NMR

Table 7
HMBC correlations of ladamycin B_3, ladamycin D_3, 5-hydroxy-100-1, 12b-derhodinosyl-urdamycin G and lourdamycin

Position	Ladamycin B_3	Ladamycin D_3	5-Hydroxy-100-1	12b-Derhodinosyl-urdamycin G	Lourdamycin
2-H_{eq}	1, 2, 4	1, 3, 4, 12b	1, 3, 3-CH_3, 4, 12b	1, 3, 4	1, 3, 4
2-H_{ax}	1, 3	1, 3	1, 3, 3-CH_3	1, 3, 3-CH_3	1, 3
3-CH_3	2, 3, 4	1, 2, 3, 4	1, 2, 3, 4	1, 2, 3, 4	2, 3, 4
4-H_{eq}	2, 3, 4a, 5, 12b	–	2, 3, 3-CH_3, 4a, 5, 12b	2, 3, 3-CH_3, 4a	2, 3, 4a, 5
4-H_{ax}	3, 4a, 12b	–	3, 3-CH_3, 4a, 5, 12b	3, 3-CH_3, 4a	4a, 5
5-H	4, 6a, 12b	6a, 12b	–	6a, 12b	–
5-OH	–	–	–	–	4a, 5, 5a
6-H	4a, 7, 12a	4a, 7, 12a	4a, 5, 6a, 7, 12a	4a, 7, 12a	–
7-H	–	–	–	–	6, 9, 10a
8-H	–	–	–	–	1′, 6a, 10,
10-H	1′, 8, 11a	1′, 8, 9, 11a, 3″	1′, 8, 11a	1′, 8, 11a	–
10-OH	–	–	–	–	9, 10, 10a
11-H	7a, 9, 12	–	7a, 9, 12	7a, 9, 12	–
12-H	–	–	–	–	1, 4a, 5a, 11
5″-H	–	3″, 4″, 6a″, 10a″	–	–	–
7″-H	–	10a″, 9″	–	–	–
8″-H	–	6a″, 10″	–	–	–
9″-H	–	7″, 10a″	–	–	–
10″-H	–	6a″, 8″, 4″	–	–	–
1′-H	8, 9, 10	9	9, 10	8, 9, 10, 2′, 3′, 5′	8, 9
2′-H_{eq}	3′, 4′	3′, 4′	3′, 4′	3′, 4′	3′, 4′
2′-H_{ax}	–	–	9, 1′	9, 1′, 3′,	1′
3′-H	–	–	–	1A, 4′	–
4′-H	1A, 3′, 5′, 6′	1A, 3′, 5′, 6′	3′, 5′, 6′	3′, 5′, 6′	–
5′-H	–	–	–	–	–
6′-CH_3	3′, 4′, 5′	4′, 5′	3′, 5′	4′, 5′	3′, 4′, 5′
1A-H	4′	4′	3′, 3A, 5A	3′, 3A, 5A	5A
2A-H_{eq}	1A, 3A, 4A	1A, 3A, 4A	1A, 4A	–	–
2A-H_{ax}	1A, 3A	1A, 3A	–	–	–
3A-H_{eq}	–	–	–	–	–
3A-H_{ax}	–	–	–	1A	–
4A-H	3A	3A, 5A, 6A	4A, 6A	–	–
5A-H	–	–	–	1A, 4A, 6A	–
6A-CH_3	4A, 5A	4A, 5A	4A, 5A	4A, 5A	4A, 5A
1B-H	3A, 3B, 5B	3A, 5B	–	–	–
2B-H_{eq}	–	–	–	–	–
2B-H_{ax}	–	–	–	–	–
3B-H_{eq}	–	–	–	–	–
3B-H_{ax}	–	–	–	–	–
4B-H	–	–	–	–	–
5B-H	4B, 6B	4B, 6B	–	–	–
6B-CH_3	4B, 5B	4B, 5B	–	–	–

data (HMBC, ^{13}C chemical shifts) clearly reveal the 1,4-linkage of the two D-olivose building blocks.

From these results we conclude that LanGT1 is a D-olivosyltransferase, which links a second D-olivose to the 4-OH group of another D-olivose, whereas LanGT4 is an L-rhodinosyltransferase, which attaches an L-rhodinose to the 3-OH group of a D-olivose. These functions are consistent with the prediction from the sequence comparison of the landomycin with the urdamycin glycosyltransferases and their proposed involvement in the hexasaccharide biosynthesis of landomycin. Especially LanGT4 displayed relaxed substrate specificity and was able to glycosylate various alcohol substrates at reasonable rates, showing the potential of glycosyltransferases to design and create new oligosaccharide structures.

4. Significance

Glycosyltransfer is an important step in the biosynthesis of antibiotics and is often needed to confer biological activity to a compound. We used two glycosyltransferase gene mutant strains derived from the urdamycin producer *S. fradiae* Tü2717 as hosts to analyze the expression of glycosyltransferase genes from the landomycin biosynthetic gene cluster of *S. cyanogenus* S136. A versatile new cloning system was implemented to facilitate the construction of gene cassettes. The production of several novel metabolites at high efficiency proves again that glycosyltransferases are useful tools in combinatorial biosynthesis due to their substrate flexibility. As shown by the production of ladamycin B_3 this technique is not restricted

to the addition of single sugar moieties but can be used to design and generate larger deoxysugar moieties. Combined with the recently gained knowledge on deoxysugar biosynthetic genes, this will further extend the possibilities of combinatorial biosynthesis.

5. Materials and methods

5.1. Bacterial strains and culture conditions

S. fradiae Tü2717 was obtained from the Department of Microbiology, University of Tübingen [12], Germany, *S. fradiae* BF-1-1 was obtained from B. Faust, Pharmaceutical Biology, University of Tübingen, Germany [15]. Conditions of growth were as described [16]. *E. coli* XL1 blue MRF' from Stratagene was used as host for plasmid propagation. Prior to transformation of *S. fradiae* plasmids were passed through *E. coli* ET12567 (*dam*-, *dcm*-, *hsds*-, *Cm*+) to generate demethylated DNA [42].

5.2. General genetic manipulation, DNA sequencing/sequence analysis and PCR

Routine methods were performed as described [43]. Transformation of *S. fradiae* strains was performed according to standard procedures [44]. Southern hybridization, sequencing and enzymatic manipulation of DNA were carried out according to manufacturer's directions (Amersham-Pharmacia Biotech, Roche Diagnostics, Stratagene, Promega). Oligonucleotide primers were purchased from Amersham-Pharmacia Biotech. PCR was performed using a GeneAmp PCR System 2400 (Applied Biosystems) with similar conditions as described [16]. For amplification of the different glycosyltransferase genes together with their own ribosomal binding site suitable cosmids or plasmids from the urdamycin or landomycin gene clusters were used as templates. Primer sequences are given in Table 3. The amplified fragments were cloned into pMUNII except for *lanGT2*, which was cloned into pMUNI. Nucleotide sequences were determined by the dideoxy chain-termination method using automatic laser fluorescence sequencers (Vistra 725, Amersham-Pharmacia Biotech). Sequencing reactions were done using a thermosequenase cycle sequencing kit with 7-deaza-dGTP (Amersham-Pharmacia Biotech) and standard primers (M13 universal and reverse, T3, T7). Computer-aided sequence analysis was done with the DNASIS software package (version 2.1, 1995; Hitachi Software Engineering), database searches were performed with the BLAST 2.0 program [45] on the server of the National Center for Biotechnology Information, Bethesda, MD, USA.

5.3. Construction of pMUN vectors

pBluescript SK− obtained from Stratagene was used for the construction of pMUNI. Introduction of new restriction sites into the multiple cloning site was performed by ligating double-stranded oligonucleotides into suitable cut plasmids (Table 4). Double-stranded oligonucleotides were obtained by annealing two complementary single-stranded oligonucleotides. For this purpose the two single-stranded oligonucleotides were denatured by heating (98°C, 10 min) and then gradually cooled down to 45°C (non-linear, 30 min). To obtain an overhang for ligating to the appropriately cut plasmid, the single-stranded oligonucleotides were extended at the 5'-end. The sequence of new plasmids was confirmed by sequencing. For the construction of pMUNI restriction sites *Bam*HI, *Sma*I, *Pst*I, *Eco*RI, *Eco*RV and *Hin*dIII in pBluescript were replaced by *Bam*HI, *Nsi*I, *Eco*RI, *Eco*RV, *Mun*I and *Hin*dIII. For the generation of pMUNII the *Bam*HI, *Nsi*I, *Eco*RI, *Eco*RV and *Mun*I sites of pMUNI were exchanged for *Eco*RI, *Bgl*II, *Nde*I, *Nsi*I, *Nco*I and *Mun*I while deleting the *Bam*HI site. pMUNIII is similar to pMUNII but retains the *Bam*HI site from pMUNI (Table 1).

5.4. Plasmids

The bifunctional plasmid pUWL201 [17] was a kind gift of U. Wehmeier and W. Piepersberg, University of Wuppertal, Germany, and used for expression of single genes and gene cassettes in *S. fradiae*. The construction of the gene inactivation plasmid pKC-12-B2 and of plasmids pMUNurdGT1a, pMUNurdGT1b and pMUNurdGT1c has been described [16].

5.5. Production, purification, structure elucidation and chemical analysis of urdamycins and novel hybrid compounds

Production, purification, TLC conditions, and HPLC-UV/Vis conditions for urdamycin derivatives were essentially as described previously [16]. For HPLC-electrospray ionization (ESI)-MS analysis separation was performed on an HP1110 (Hewlett-Packard) with a HP-ODS-Hypersil (100×2.1 mm; 5 μm) at a flow rate of 0.1 ml/min, detection at 250 nm and the following gradient: 0–5 min 20% B, 5–50 min linear to 55% B, 50–60 min linear to 90% B (solvent A: 90% H_2O, 9.95% acetonitrile, 0.05% acetic acid; solvent B: 90% acetonitrile, 9.95% H_2O, 0.05% acetic acid). Mass spectra were recorded on a Bruker Esquire LC 1.6n mass spectrometer (Bruker Daltronik) equipped with an electrospray ion source (positive ion mode) (ESI) at a scan range of 50–1200 *m/z* with nominal mass resolution.

The structures of ladamycins B_2, B_3, D_3, 5-hydroxy-100-1, urdamycin Q and the rearranged lourdamycin were identified mainly by NMR spectroscopy, all these structures were also supported by MS. The instrumentation used was essentially the same as described previously [6,46], except the Varian NMR instrument, which was recently upgraded into an INOVA 400. For details, see text and Tables 5–7. The FAB mass spectra and the high resolutions were recorded at the University of South Carolina Department of Biochemistry and Chemistry facilities in Columbia, SC, USA, using a VG70SQ double focussing magnetic sector MS instrument. The APCI mass spectra were recorded at the Medical University of South Carolina regional mass spectroscopy center using a Finnigan MAT LCQ.

The structures of ladamycins A_2 and A_3 were determined by HPLC-MS (see text).

The known urdamycins mentioned here were identified by comparison with authentic samples using TLC, HPLC-UV/Vis, HPLC-MS and ^1H NMR.

Acknowledgements

We would like to thank Drs. William Cotham, Michael Walla (University of South Carolina, Department of Bio-

chemistry and Chemistry) and Mr. Uwe Rix (Medical University of South Carolina) for the MS data. The work was supported by a Grant of the European Union (QLK3-CT-1999-00095) to A.B. and by grants of the South Carolina Department of Higher Education (2000–2001), in part by MUSC Institutional Research Funds of 1999-00, and the US Department of Defense to J.R.

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Oviedomycin, an Unusual Angucyclinone Encoded by Genes of the Oleandomycin-Producer *Streptomyces antibioticus* ATCC11891

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JOURNAL OF NATURAL PRODUCTS®

Reprinted from
Volume 65, Number 5, Pages 779–782

Oviedomycin, an Unusual Angucyclinone Encoded by Genes of the Oleandomycin-Producer *Streptomyces antibioticus* ATCC11891

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Received November 5, 2001

Our investigations on the discovery of novel natural metabolites using type II polyketide synthase gene probes (*act*I/III) yielded an unusual angucyclinone, oviedomycin (**2**), when applied to the oleandomycin (**1**) producer *Streptomyces antibioticus* ATCC11891. The novel natural product was produced using *S. albus* R^-M^- as a host strain, into which a cosmid containing the oviedomycin gene cluster was transformed. Its structure was elucidated by NMR spectroscopy and mass spectrometry.

Genes that are unexpressed under normal conditions as well as genes from noncultivatable organisms are discussed in the context of drug discovery strategies. The search for such genes in bacteria of the genus *Streptomyces* seems profitable, since they not only produce the most biologically active natural products but are also known for their large genome, which often contains far more biosynthetic genes than necessary to encode the biosynthesis of the compounds normally produced by a certain strain. In particular, polyketide synthase (PKS) encoding genes are interesting, since polyketides represent perhaps the largest and structurally most diverse group of natural products, often displaying interesting biological and pharmaceutical activities.[1−5] PKS genes have even been found in bacterial strains that are not known to produce polyketides. For example, polyketide synthase genes were recently discovered in various *Mycobacteria*, and in one case an interesting polyketide natural product was already identified.[6−8] Thus, the search for PKS encoding genes and novel polyketides is promising. Among the various PKS, one may distinguish between (i) type I PKS, which typically catalyze the biosynthesis of modularly assembled macrolactones (e.g., erythromycin or oleandomycin) or polyethers;[2−5,9,10] (ii) type II PKS, which catalyze the synthesis of multicyclic, mostly aromatic, compounds that are often pigments (e.g., anthracyclines, tetracyclines, angucyclines);[2−5,11−15] and (iii) type III PKS,[16−18] which occur in plants (chalcone synthases) and were recently also discovered in bacteria. Usually, only one of these three PKS systems is described in context with a certain producer of natural compounds, although type II PKS genes that have been found to encode the biosynthesis of spore pigments of Streptomycetes[19] should exist in most strains of this genus.

Streptomyces antibioticus ATCC11891 is known as an oleandomycin (**1**) producer, which is a macrolide antibiotic, biosynthesized by a modular type I PKS. The oleandomycin gene cluster, which contains three genes for a modular polyketide synthase, has been cloned and characterized.[20−26] In this paper, we describe the identification of type II PKS genes in *S. antibioticus* ATCC11891, which code for a previously unknown, usually not produced, aromatic metabolite that may be a spore pigment.

To identify an aromatic polyketide gene cluster in *S. antibioticus* ATCC11891, a cosmid library of the total DNA of this microorganism,[26] constructed in the bifunctional (*Streptomyces*−*E. coli*) cosmid pKC505,[27] was hybridized with the *act*I/III probes from the actinorhodin gene cluster in *S. coelicolor*.[28] Both probes are part of a typical type II PKS gene cluster, with the *act*I probe containing part of the minimal polyketide synthase PKS genes and the *act*III probe containing a ketoreductase gene.[29] Two different clusters with putative type II PKS genes were identified: (a) Cluster I, cosmid clones that hybridize with the *act*I/III probes in a single 8 kb *Bam*HI band; five overlapping cosmid clones were isolated as part of this group, cosAB3, cosAB4, cosAB16, cosAB50, and cosAB51 (Figure 1); and (b) Cluster II, clones hybridizing with the *act*I probe in a 1.8 kb *Bam*HI fragment and with the *act*III region in a 8 kb *Bam*HI fragment. From this cluster, four overlapping cosmid clones were isolated, cosAB18, cosAB26, cosAB36, and cosAB38. All cosmids belonging to Clusters I and II were analyzed for their ability to produce aromatic polyketides. For this, they were used to independently transform protoplasts of *S. lividans* TK21[30] as well as *S. albus* R^-M^-.[31] Transformants were selected for their resistance to apramycin (25 μg/mL final concentration) on R5 (=R2YE)[30] medium and were screened for the production of pigments. Neither the control nor any of the cosmids belonging to Cluster II induced the production of a pigment in either of the *Streptomyces* hosts. However, in the case of cosmids belonging to Cluster I, some (cosAB3, cosAB4, cosAB16, and cosAB50) led to the production of a red-brown pigment both in *S. albus* and in *S. lividans*. While the pigment in those *Streptomyces* strains harboring cosAB16 seemed to remain in the colony, the pigment was diffusible in the case of those strains harboring cosAB3 or cosAB4 (more visible with cosAB3). The pigment produced by the strain harboring cosAB3 was isolated from bulk cultures to elucidate its structure.

The structure of the pigment, which was subsequently named oviedomycin, was elucidated using NMR and mass spectroscopy. Oviedomycin (**2**) has a molecular formula of

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10.1021/np010555n CCC: $22.00
Published on Web 04/25/2002

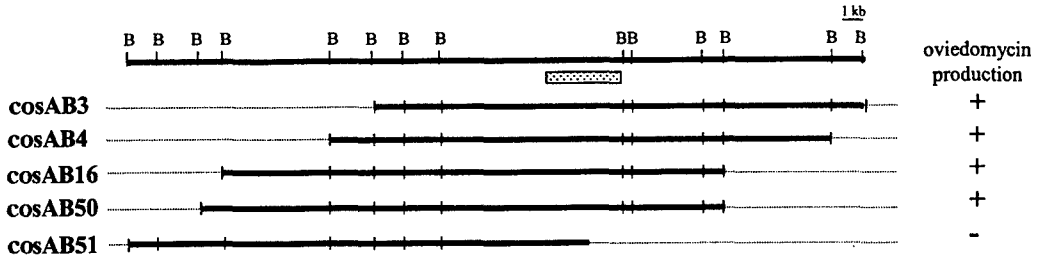


Figure 1. Schematic representation of overlapping cosmids from polyketide Cluster 1 region of *S. antibioticus*. B = *Bam*H1; shaded square = *actI*/III hybridizing region.

Table 1. NMR Data of Oviedomycin (**2**) (DMSO-d_6, 400 and 100.7 MHz, respectively)

position	δ_H mult. (*J* in Hz)	δ_C	H,H-COSY couplings	HMBC couplings
1		178.5		
OH-2	n.o.[a]	158.0 (181.0)[b]		
3	4.05 q (6)[c]	119.2		
4		182.9		
4a		139.4		
5	7.62 s	118.0		120.6 (^3J)
				123.8 (^3J)
				182.9 (^3J)
				139.4 (^2J)
				163.7 (^2J)
				178.5 (^4J)[d]
				189.9 (^4J)[d]
OH-6	n.o.[a]	163.7		
6a		120.6		
7		189.9		
7a		115.8		
OH-8	11.53 br s	160.5		
9	7.39 d (8)	123.7	7.50; 7.80	115.8 (^3J)
				118.3 (^3J)
				160.5 (^2J)
				135.6 (^4J)[d]
				189.9 (^4J)[d]
10	7.80 dd (8, 8)	137.5	7.39; 7.50	135.6 (^3J)
				160.5 (^3J)
				118.3 (^2J)
				123.7 (^2J)
				115.8 (^4J)[d]
				186.3 (^4J)[d]
11	7.50 d (8)	118.3	7.39; 7.80	115.8 (^3J)
				123.7 (^3J)
				186.3 (^3J)
				137.5 (^2J)
				160.5 (^4J)[d]
				189.9 (^4J)[d]
11a		135.6		
12		186.3		
12a		138.7		
12b		123.8		
13	1.93 s	8.3		158.0 (^3J)
	1.25 d (6)[c]			(181.0) (^3J)
				182.9 (^3J)
				119.2 (^2J)

[a] Not observed. [b] Due to keto–enol tautomerism. [c] Keto form. [d] Weak coupling signal.

$C_{19}H_{10}O_7$ (350.2874 g/mol) evident from the ^{13}C NMR data (19 carbons) and especially from the molecular ion peak of the HREIMS (*m/z* 350, 60%; calcd for $C_{19}H_{10}O_7$; *m/z* 350.0427, found *m/z* 350.0433). The ^1H NMR data revealed only an aromatic ABC system, an additional aromatic singlet, and a sp^2-bound methyl group. Only one OH signal was observed, although the structure (**2**, see below) demands three; two of them were evidently exchanged. The large number of quaternary carbons (14 out of 19) complicated the structure determination. However, the long-range couplings observed in the HMBC spectrum (see Table 1), particularly the long-range couplings of the diagnostic H-5 singlet (δ 7.72 s) with seven different carbons, permitted

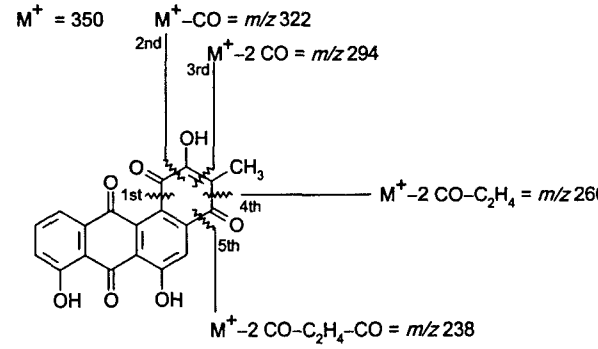


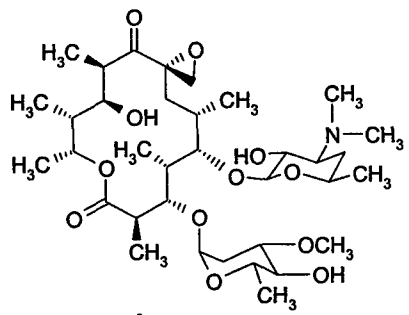
Figure 2. EIMS fragmentation of oviedomycin (**2**).

the combination of all determined structural elements as in structure **2**. For instance, the anellation of ring D followed from the fact that both H-5 and H-9 showed a small, diagnostic $^4J_{C-H}$ coupling to the same carbonyl (C-7, δ 189.9), and H-11 showed a large $^3J_{C-H}$ coupling to C-12 (δ 186.3) and a small $^4J_{C-H}$ coupling to C-7 (δ 189.9). Independent confirmation of the substitution pattern of ring A came from the EIMS, showing the consecutive fragmentation of the C-12b/C-1, C-1/C-2, C-2/C-3, C-3/C-4, and C-4/C-4a bonds. This fragmentation was verified by accurate mass determination of the resulting MS peaks (see Experimental Section and Figure 2). Thus, oviedomycin (**2**) is an angucyclinone with novel structural features, particularly the two quinone systems and the unusually substituted ring A. This oxygen-rich ring A bears three carbonyls, one of which (C-2) is predominantly enolized, although a small amount of the keto form (ca. 10%) was also visible in the NMR spectra, as indicated by several signals (e.g., δ_C of C-2: 158.0 and 181.0; δ_H of CH_3-3: 1.93 s and 1.25 d; δ_H of H-3: 4.05 q).

Considering the typical biosynthetic incorporation of acetate units into the angucyclinone backbone,[14,15] at least three of the oxygens (O-2, O-3, and O-12) may be introduced by oxygenases, while the remaining four might stem from acetate. Oxygenation at the C-4 position is unprecedented among angucyclinones. Thus, the investigation of the oviedomycin biosynthetic gene cluster, planned for the near future, will reveal novel genes, which may be useful for combinatorial biosynthetic studies.

The experiments described here show that it is profitable to screen and heterologously express type II PKS genes to stimulate the production of undiscovered novel multicyclic, aromatic compounds. The results suggest that oviedomycin (**2**) is a product of heterologously expressed *S. antibioticus* genes; however, it cannot be ruled out that the host strain provided some of the enzyme activities, which may have contributed to the formation of **2**. Additional experiments using various growth conditions showed that traces of oviedomycin (**2**) could also be detected in *S. antibioticus* when grown on solid agar using GAE medium,[32] and it seems that **2** confers a brown pigmentation to the colonies. However, this is hard to observe, since *S. antibioticus* also

produces large amounts of melanin that masks the oviedomycin pigmentation.



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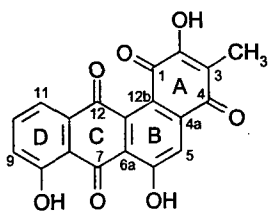

2

Experimental Section

General Experimental Procedures. UV spectra were recorded on a Beckman DU 650 spectrophotometer, and the IR spectrum was obtained from a pure sample on KCl disks in a Mattson Genesis II FT FT-IR spectrometer. NMR spectra were recorded in DMSO-d_6 on Varian Inova 400 and Bruker DMX 500 NMR instruments at 400 and 500 MHz, respectively, for ^{1}H and 100.6 and 125.7 MHz, respectively, for ^{13}C, using 1D and 2D homo- and heteronuclear correlation experiments (^{1}H, ^{13}C, DEPT, H,H-COSY, HSQC, and HMBC). Electron-impact mass spectrometry (EIMS) was carried out using a VG 70SQ double-focusing magnetic sector mass spectrometer with sample introduction by direct exposure probe and an ionization energy of 70 eV. HPLC was performed on a Waters HPLC system (Delta 600, M32 add-on single system, with a photodiode array detector model 996), using the columns and guard columns described below.

Bacterial Strains, Plasmids, and Gene Probes. For constructing a cosmid gene library of total DNA of *S. antibioticus*,[26] the bifunctional (*Streptomyces–E. coli*) cosmid, pKC505,[33] was used. The obtained cosmids were hybridized with the *actI*/III probes from the actinorhodin gene cluster in *S. coelicolor*.[28] The *act*I probe corresponds to a 2.2 kb *Bam*HI fragment from pIJ2345 that contains part of the polyketide synthase genes, and the *act*III probe corresponds to a 1.1 kb *Bam*HI fragment from pIJ2363 that contains the ketoreductase gene.[29] As host strains for transformation, *S. lividans* TK21 and *S. albus* R$^-$M$^-$[31] were used. Protoplast transformations were according to standard procedures.[31,34] In control experiments, cosmid pKC505[27] was transformed.

Cultivation and Fermentation. For the production of oviedomycin, the strain *Streptomyces albus* R$^-$M$^-$ (cosAB3) was incubated in eight 2 L Erlenmeyer flasks each containing 400 mL of SCM medium,[34] with glycerol substituting for starch, and supplemented with 2.5 μg/mL of apramycin, for 3 days at 30 °C and 250 rpm.

Isolation of Oviedomycin (2). The cultures were centrifuged, and the supernatants were filtered, adjusted to pH 5.5 with formic acid, and applied to a solid-phase extraction cartridge (Supelclean LC-18; 10 g; Supelco). The cartridge was eluted with methanol and 0.1% trifluoroacetic acid in water, using a linear gradient from 0 to 100% methanol in 60 min, at 10 mL/min, taking fractions every 5 min. The eluate collected between 45 and 55 min contained most of the compound, as assessed by HPLC analysis of the fractions. The material in these fractions was dried in vacuo and redissolved in a small amount of dimethyl sulfoxide. The product was purified by preparative HPLC in a μBondapak C_{18} radial compression cartridge (PrepPak Cartridge; 25 by 100 mm; Waters). An isocratic elution with a mixture of acetonitrile and 0.1% trifluoroacetic in water (55:45), at 10 mL/min, allowed the separation of **2** as a pure peak, which was collected and dried in vacuo, yield 12.5 mg/L, obtained as an amorphous orange-red solid.[35]

Characterization of Oviedomycin (2): UV [MeOH] λ_{max} (ϵ) 473 (3600), 275 (12800), 218 (12300) nm; [MeOH–HCl] λ_{max} (ϵ) 433 (4400), 320 sh (4300), 277 (12800), 220 (12500) nm; [MeOH–NaOH] λ_{max} (ϵ) 522 (4100), 372 (6700), 279 (12700), 227 (12800) nm; IR ν_{max} (KCl) 3290, 2929, 2864, 7143sh, 1676, 1630, 1596, 1576, 1534, 1476, 1409, 1353, 1267, 1201, 1031 cm^{-1}; ^{1}H NMR, ^{13}C NMR, and observed COSY and HMBC couplings, see Table 1; HREIMS *m/z* 350 (M$^+$, 350.0433, calcd for $C_{19}H_{10}O_7$, 350.0427, 60), 322 (M − CO, 322.0478, calcd for $C_{18}H_{10}O_6$, 322.0477, 100), 294 (M − 2CO, 294.0525, calcd for $C_{17}H_{10}O_5$, 294.0528, 40), 266 (M − 2CO − C_2H_4, 266.0218, calcd for $C_{15}H_6O_5$, 266.0215, 25), 238 (M − 2CO − C_2H_4 − CO, 238.0267, calcd for $C_{14}H_6O_4$, 238.0266, 20); see also Figure 2.

Acknowledgment. This work was partly supported by grants from the U.S. Department of Defense, awarded to J.R., and the Spanish Ministry of Science and Technology (Plan Nacional en Biotecnología BIO2000-0274), awarded to J.A.S.

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Chemistry & Biology

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Research Paper

Identification of a sugar flexible glycosyltransferase from *Streptomyces olivaceus*, the producer of the antitumor polyketide elloramycin

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Received 25 October 2000; accepted 2 February 2001
First published online 22 February 2001

Abstract

Background: Elloramycin is an anthracycline-like antitumor drug related to tetracenomycin C which is produced by *Streptomyces olivaceus* Tü2353. Structurally is a tetracyclic aromatic polyketide derived from the condensation of 10 acetate units. Its chromophoric aglycon is glycosylated with a permethylated L-rhamnose moiety at the C-8 hydroxy group. Only limited information is available about the genes involved in the biosynthesis of elloramycin. From a library of chromosomal DNA from *S. olivaceus*, a cosmid (16F4) was isolated that contains part of the elloramycin gene cluster and when expressed in *Streptomyces lividans* resulted in the production of a non-glycosylated intermediate in elloramycin biosynthesis, 8-demethyl-tetracenomycin C (8-DMTC).

Results: The expression of cosmid 16F4 in several producers of glycosylated antibiotics has been shown to produce tetracenomycin derivatives containing different 6-deoxysugars. Different experimental approaches showed that the glycosyltransferase gene involved in these glycosylation events was located in 16F4. Using degenerated oligoprimers derived from conserved amino acid sequences in glycosyltransferases, the gene encoding this sugar flexible glycosyltransferase (*elmGT*) has been identified. After expression of *elmGT* in *Streptomyces albus* under the control of the erythromycin resistance promoter, *ermEp*, it was shown that *elmG* can transfer different monosaccharides (both L- and D-sugars) and a disaccharide to 8-DMTC. Formation of a diolivosyl derivative in the mithramycin producer *Streptomyces argillaceus* was found to require the cooperative action of two mithramycin glycosyltransferases (MtmGI and MtmGII) responsible for the formation of the diolivosyl disaccharide, which is then transferred by ElmGT to 8-DMTC.

Conclusions: The ElmGT glycosyltransferase from *S. olivaceus* Tü2353 can transfer different sugars into the aglycon 8-DMTC. In addition to its natural sugar substrate L-rhamnose, ElmGT can transfer several L- and D-sugars and also a diolivosyl disaccharide into the aglycon 8-DMTC. ElmGT is an example of sugar flexible glycosyltransferase and can represent an important tool for combinatorial biosynthesis.

Keywords: Polyketide; Anticancer; Deoxyhexose; Anthracycline; Mithramycin

1. Introduction

A number of bioactive compounds contain sugars attached to their aglycons. Usually, glycosylation occurs as late steps in biosynthesis and the addition of the respective sugars is important and, in many cases, essential for biological activity of the compounds. Most of these sugars belong to the large family of the 6-deoxyhexoses (6-DOH) which form part of a great number of natural products and at least 80 different 6-DOH have been identified in different metabolites [1–3]. In the last years, a number of deoxysugar biosynthetic pathways from antibiotic-producing organisms are increasingly being reported [3], providing the information needed to design new gene combinations directing the biosynthesis of different 6-DOHs. In

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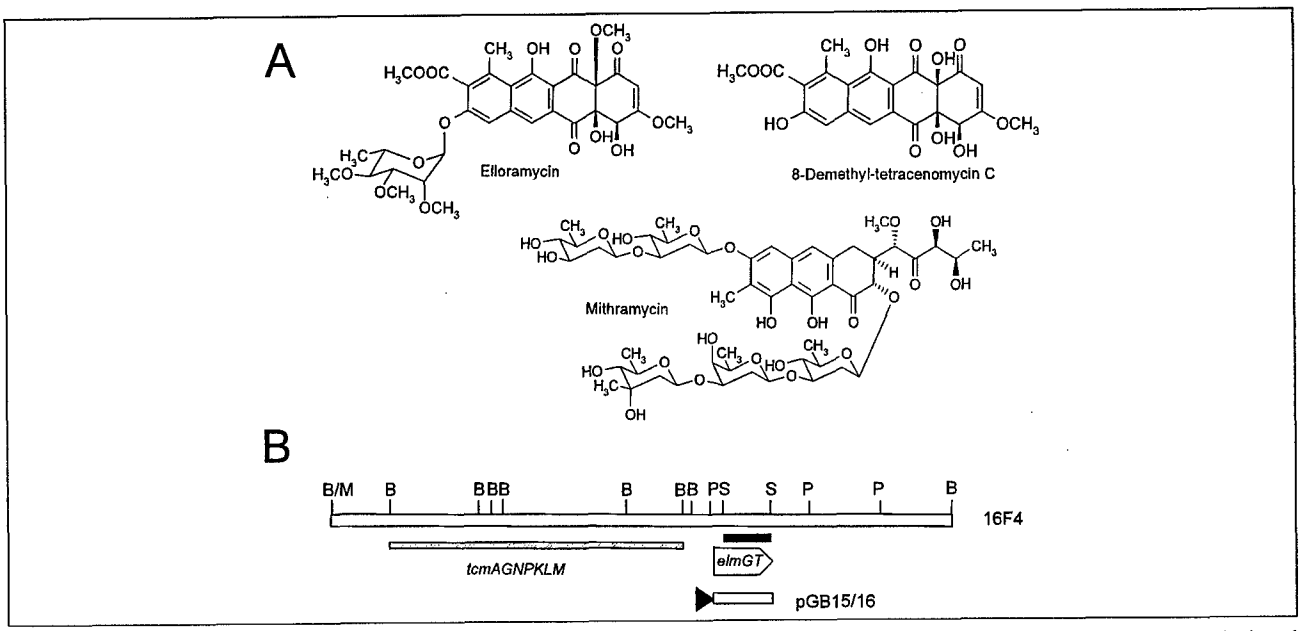

Fig. 1. (A) Structures of elloramycin, 8-DMTC and mithramycin. (B) Schematic representation of the restriction map of cosmid 16F4: the shadowed bar indicates the region that has been shown to hybridize with the *tcmAGNPKLM* genes of the tetracenomycin C gene cluster [17]. The black bar shows the location of the fragment hybridizing against the 350 bp PCR probe. The black triangle represents the *ermEp*. B, *Bam*HI; M, *Mbo*I; P, *Pst*I; S, *Sal*I.

addition, several glycosyltransferase genes have been cloned and characterized [4–13], supplying the necessary tools for the potential generation of new glycosylated bioactive compounds.

Elloramycin is an anthracycline-like antitumor drug produced by *Streptomyces olivaceus* Tü2353 [14]. It is active against Gram-positive bacteria and also exhibits antitumor activity [15]. Structurally, elloramycin belongs to the large and important family of the aromatic polyketides [16]. Its aglycon closely resembles tetracenomycin C, but has an additional C-12a-*O*-methyl group and, in contrast to tetracenomycin C, is glycosylated with a permethylated L-rhamnose residue at the C-8 hydroxyl group (Fig. 1A). Not too much information exists about genes involved in elloramycin biosynthesis. Using a cosmid library of chromosomal DNA from *S. olivaceus*, several overlapping cosmid clones were isolated by probing this cosmid library with two polyketide synthase genes of the tetracenomycin cluster (*tcmK* and *tcmL*) [17]. Two positive cosmid clones (16F4 and 4F5) were characterized in some detail. Different tetracenomycin C genes were used as probes to detect the presence of homologous elloramycin genes in these cosmids (Fig. 1B). The conclusion was that basically the sequential order of the *tcm* and *elm* genes appeared to be very similar [17]. Expression of 16F4 in *Streptomyces lividans* TK64 resulted in the production of several putative intermediates in elloramycin biosynthesis, 8-demethyl-tetracenomycin C (8-DMTC; Fig. 1A), a non-glycosylated intermediate, being the major compound. It was therefore assumed that 16F4 should contain all genes necessary for the biosynthesis of the polyketide moiety of elloramycin and should probably lack some of the genes required for L-rhamnose biosynthesis or its transfer to the aglycon.

Interestingly, 16F4 has been shown to be a good genetical tool for the generation of novel compounds by combinatorial biosynthesis. Novel hybrid compounds were obtained after introducing 16F4 into different producer strains. These were hybrid compounds between urdamycin and elloramycin [18], mithramycin and elloramycin [19] and oleandomycin and elloramycin [20]. All these compounds have a common feature: they share the aglycon (8-DMTC encoded by 16F4) and contain different sugar moieties synthesized by the host strain. Here we report experimental evidence demonstrating the presence of a sugar flexible glycosyltransferase in 16F4, its cloning and expression and evidence of the sugar flexible capability of this enzyme.

2. Results and discussion

2.1. *cos16F4 contains a gene encoding a glycosyltransferase responsible for the formation of glycosylated tetracenomycins*

The formation of different glycosylated tetracenomycins after expression of 16F4 into several producer organisms raised a question about the genetic location of the gene encoding the glycosyltransferase responsible for sugar transfer into the aglycon: would it be coded by 16F4 or would it be a glycosyltransferase present in the transformation host? To discern between these two possibilities we

carried out several experiments using several hosts with different genetic backgrounds (Table 1). When 16F4 was transformed into the mithramycin producer, *Streptomyces argillaceus*, four compounds were produced by the recombinant strain: the elloramycin aglycon 8-DMTC and three glycosylated derivatives. Two of them contained a D-olivose or a D-mycarose moiety attached to the aglycon, 8-demethyl-8-β-D-olivosyl-tetracenomycin C (DOLV-TCM) and 8-demethyl-8-β-D-mycarosyl-tetracenomycin C (DMYC-TCM). The third one contained a diolivosyl disaccharide attached to the aglycon, 8-demethyl-8-β-D-diolivosyl-3′-1″-D-β-tetracenomycin C (DDIOLV-TCM). The purification and structural elucidation of these compounds was previously carried out [19] and from that moment it was possible to identify these compounds in the different recombinant strains by comparison of their high performance liquid chromatography (HPLC) mobility and absorption spectra with pure samples.

To verify if a glycosyltransferase from the host strain was responsible for the sugar transfer, *S. argillaceus* wild type strain was transformed with pWHM1026Δ*tcmO*. This plasmid contains the entire tetracenomycin C gene cluster with the exception of the *tcmO* gene that codes for a 8-*O*-methyltransferase and therefore directs the production of 8-DMTC. The resultant recombinant strain produced 8-DMTC but none of the glycosylated compounds were formed. Since in this strain the aglycon was produced and the sugars to be transferred (D-olivose and D-mycarose) were synthesized by the host strain, the absence of glycosylated tetracenomycins was a clear indication that the glycosyltransferase was absent from this recombinant strain and therefore from the *S. argillaceus* host genome. A similar result and conclusion was obtained by feeding *S. argillaceus* wild type strain with 8-DMTC since no glycosylated tetracenomycin derivatives were generated. These results strongly suggested that the glycosyltransferase should be coded by 16F4.

Another strong indication that 16F4 harbors the glycosyltransferase gene came from experiments in which 16F4 was used to transform the *S. argillaceus* M3ΔMG mutant. This is a mithramycin non-producing mutant in which a DNA fragment has been deleted by gene replacement eliminating a region containing four mithramycin glycosyltransferases (*mtmGI*, *mtmGII*, *mtmGIII* and *mtmGIV*) and two methyltransferases (*mtmMI* and *mtmMII*) from the chromosome [21]. When 16F4 was transformed into the M3ΔMG mutant, three of the four compounds were produced (8-DMTC, DOLV-TCM and DMYC-TCM), DDIOLV-TCM not being detected. This demonstrates that generation of the monoglycosylated derivatives is not dependent upon the activity of any mithramycin glycosyltransferase (since none of them are present in this mutant) but it requires the presence of a glycosyltransferase gene in 16F4. Interestingly, DDIOLV-TCM was only formed in *S. argillaceus* wild type strain but not in this mutant (see below for further experiments and discussion).

The final and definitive evidence demonstrating that the glycosyltransferase is present in 16F4 and not in the host strains came from another experiment in which 16F4 was transformed into *S. lividans* harboring pFLOS. This is a plasmid that contains several genes of the mithramycin gene cluster (*mtmD*, *mtmE*, *mtmC*, *mtmU*, *mtmV*, *mtmT3* and *mtmW*) most of which were supposed to be involved in sugar biosynthesis. The recombinant strain *S. lividans* FLOS/16F4 directed the production of two of the glycosylated compounds, but not DDIOLV-TCM. Since no mithramycin glycosyltransferases are present in this host strain, this experiment was a clear and definitive proof that the glycosyltransferase is encoded by 16F4. The conclusion of all these experiments was that 16F4 must contain a gene encoding a glycosyltransferase that could transfer individually D-olivose and D-mycarose or a diolivosyl disaccharide to the 8-DMTC aglycon, providing this disaccharide is previously synthesized by the mithramycin glycosyltransferases (see below).

2.2. *Cloning of the elmGT glycosyltransferase gene*

To clone the elloramycin glycosyltransferase gene from 16F4 we initially used a hybridization approach. The

Table 1
Biosynthesis of glycosylated tetracenomycins by different recombinant strains

Host	Plasmid/cosmid constructs	Products			
		8-DMTC	DOLV-TCM	DMYC-TCM	DDIOLV-TCM
S. argillaceus wt	16F4	+	+	+	+
S. argillaceus wt	pWHM1026Δ*tcmO*	+	−	−	−
S. argillaceus M3ΔMG	16F4	+	+	+	−
S. lividans TK21	16F4	+	−	−	−
S. lividans TK21	16F4+pFLOS	+	+	+	−
S. argillaceus M3G1	16F4	+	+	+	−
S. argillaceus M3G2	16F4	+	+	+	−
S. argillaceus M3ΔMG	pGB15+pEPG1	+	+	+	−
S. argillaceus M3ΔMG	pGB15+pEPG2	+	+	+	−
S. argillaceus M3ΔMG	pGB15+pEPG12	+	+	+	+

8-DMTC, 8-demethyl-tetracenomycin C; DOLV-TCM, 8-demethyl-8-β-D-olivosyl-tetracenomycin C; DMYC-TCM, 8-demethyl-8-β-D-mycarosyl-tetracenomycin C; DDIOLV-TCM, 8-demethyl-8-β-D-diolivosyl-3′-1″-D-β-tetracenomycin C.

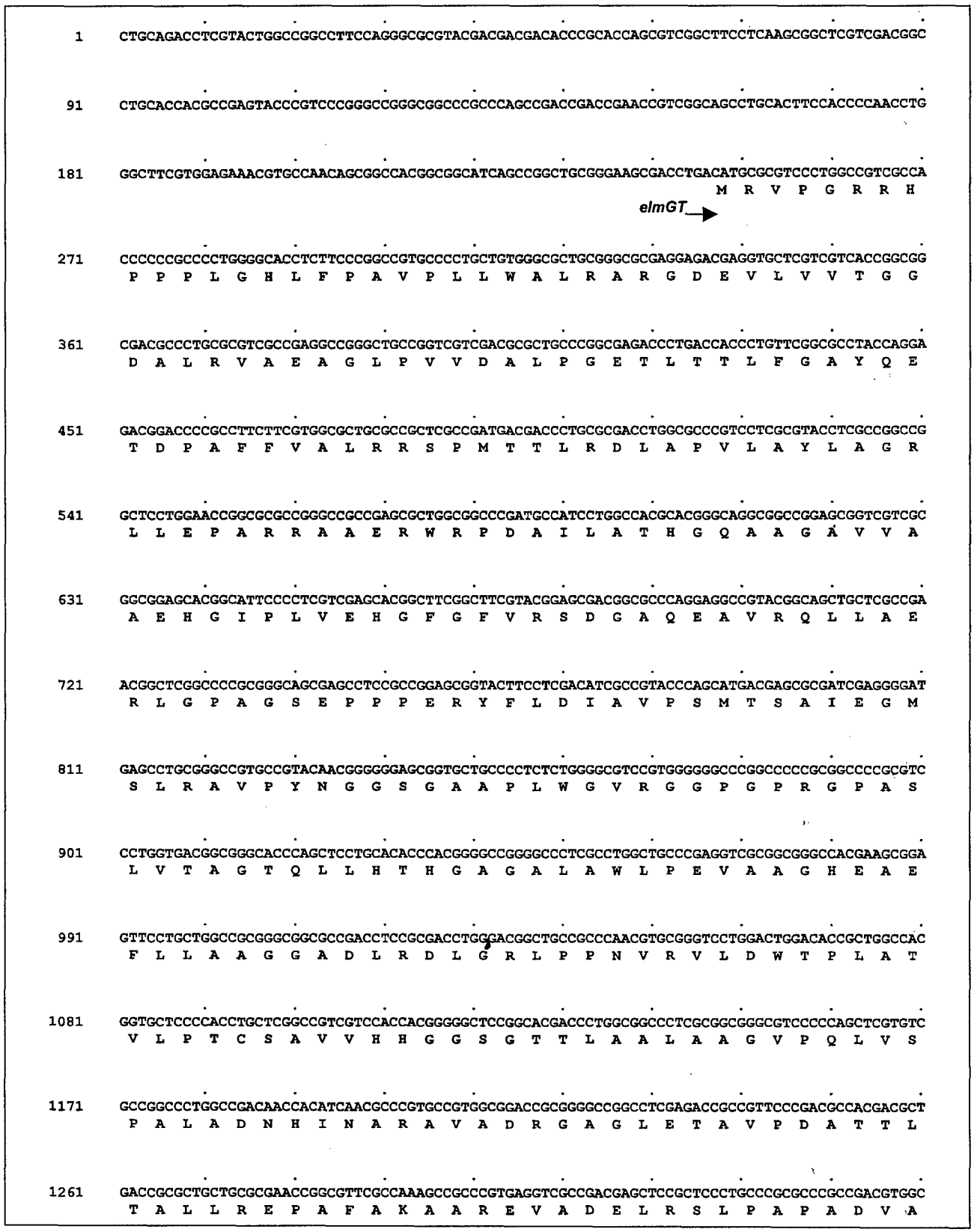

Fig. 2. Nucleotide sequence of the 1850 bp DNA region sequenced containing elmGT. The complete nucleotide sequence of the fragment is shown with the deduced amino acid sequence of elmGT in a single letter code. The sequence has been deposited in GenBank under the accession number AJ300305.

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1351   CGCCCGTCTGCACACCGCGTTCGGCCTCCCGACCACTCAAGGAGACGCATGACCACCCCTTCACCCACGCCACCGCTCGCGGCCGCCGAG
         A  R  L  H  T  A  F  G  L  P  T  T  Q  G  D  A  *

1441   GTGGCCGCCGCCGCGGGACTCCAGCAGCGGACCTGCTCGCGGTGCTCGACCGGGTCGGCCTGGAACCCGCCGTCGCGTTCCTCGTCCATG

1531   ACCTCACGGCCCGCTGCGACACCCCCGACAACCCGGACGCGGCCCGGATCGGCCTCGCCGTCGAGCACAGCGGACACCGGACCGAGCGGG

1621   TCCTCGCCGTGCGCAAGGGCGAGCCCGTCCGGGTGGACGAGAAGACGGCGGGTCCGCCTCCCGTACGTCTGACCTTCGACCTCGCCGACC

1711   TGGTCCGTGGCGTGTACGGACCGCCGCCCGGTCCCGGAGCCGGTCTGTTCCGGGTCGAACGCGACGACGCCTGGTTCGTCAAGAACGCCG

1801   ACGACGCCGAGCCCTTCCGGATCTTCGAGTCGTACATGCGCGCCGTCGAC    1850
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Fig. 2 (*continued*).

mtmGI glycosyltransferase gene from the mithramycin cluster was used as a probe against *Bam*HI-digested cosmid 16F4 DNA; however, no clear hybridization signal was observed in spite of using different stringency conditions. We therefore used another approach by using polymerase chain reaction (PCR) and two oligoprimers derived from consensus amino acid sequences obtained after comparison of known glycosyltransferases from antibiotic-producing microorganisms (Fig. 3A). Using this pair of oligoprimers and 16F4 DNA as template, a 350 bp PCR fragment was amplified. Preliminary partial sequence of this fragment and comparison of its deduced amino acid sequence with proteins in databases showed that it could code for an internal region of a glycosyltransferase. This PCR fragment was then used as probe to locate the glycosyltransferase gene within 16F4 and found to hybridize within a 9.5 kb *Bam*HI fragment located at the right-hand-side of 16F4 (Fig. 1B). Further subcloning and Southern analysis reduced the hybridizing region to a minimal region of 1.5 kb *Sal*I fragment (Fig. 1B). A region of 1.85 kb was sequenced and the nucleotide sequence (Fig. 2) analyzed for the presence of potential coding regions using the CODONPREFERENCE program [22]. The presence of an open reading frame (ORF) was observed (named *elmGT*) that started at an ATG codon and ended at a TGA codon. This ORF comprised 1152 nucleotides and coded for a polypeptide of 384 amino acids and had an estimated M_r of 39 674 Da. This ORF showed the characteristic high GC content of *Streptomyces* genes and the bias in the third codon position characteristic of the genes of this genus. Comparison of the deduced product of *elmGT* with proteins in databases, showed clear similarities with different glycosyltransferases. The highest scores were with SnogD, a glycosyltransferase from *Streptomyces nogalater* involved in nogalamycin biosynthesis (39.5% identity) [23], UrdGT1b and UrdGT1c, glycosyltransferases from *Streptomyces fradiae* involved in urdamycin biosynthesis (39 and 38% identity, respectively) [13], IroB a glycosyltransferase from *Salmonella typhi* (34% identity) [23], OleG2 a glycosyltransferase from *Streptomyces antibioticus* involved in oleandomycin biosynthesis (34% identity) [8], MtmGII a glycosyltransferase from *S. argillaceus* involved in mithramycin biosynthesis (34% identity) [7] and LanGT2 a glycosyltransferase from *Streptomyces cyanogenus* involved in landomycin biosynthesis (30% identity) [11]. A comparative dendrogram showing similarities between the ElmGT and other antibiotic glycosyltransferases is shown in Fig. 3B.

2.3. *Confirmation of the ElmGT sugar flexibility*

Once the elloramycin glycosyltransferase gene was identified, it was expressed in different hosts for in vivo assay of its capability to transfer several dTDP-sugars into the elloramycin aglycon. The *elmGT* gene was subcloned under the control of the erythromycin resistance promoter *ermEp* in the multicopy plasmid vector pIAGO (generating pGB15) and then together with the promoter in the integrative plasmid vector pKC796 (generating pGB16). After transforming protoplasts of *Streptomyces albus* with pGB16, clones in which the plasmid was integrated into the chromosome through the *att* integration site were identified by resistance to apramycin and verified by Southern analysis. The resultant recombinant strain (GB16) was then transformed independently with several plasmid constructs that direct the biosynthesis of different dTDP-L-sugars (Table 2). These constructs (pRHAM, pOLE and pOLV) contain different combinations of oleandrose biosynthetic genes from *S. antibioticus*, an oleandomycin producer, and direct the biosynthesis of dTDP-L-

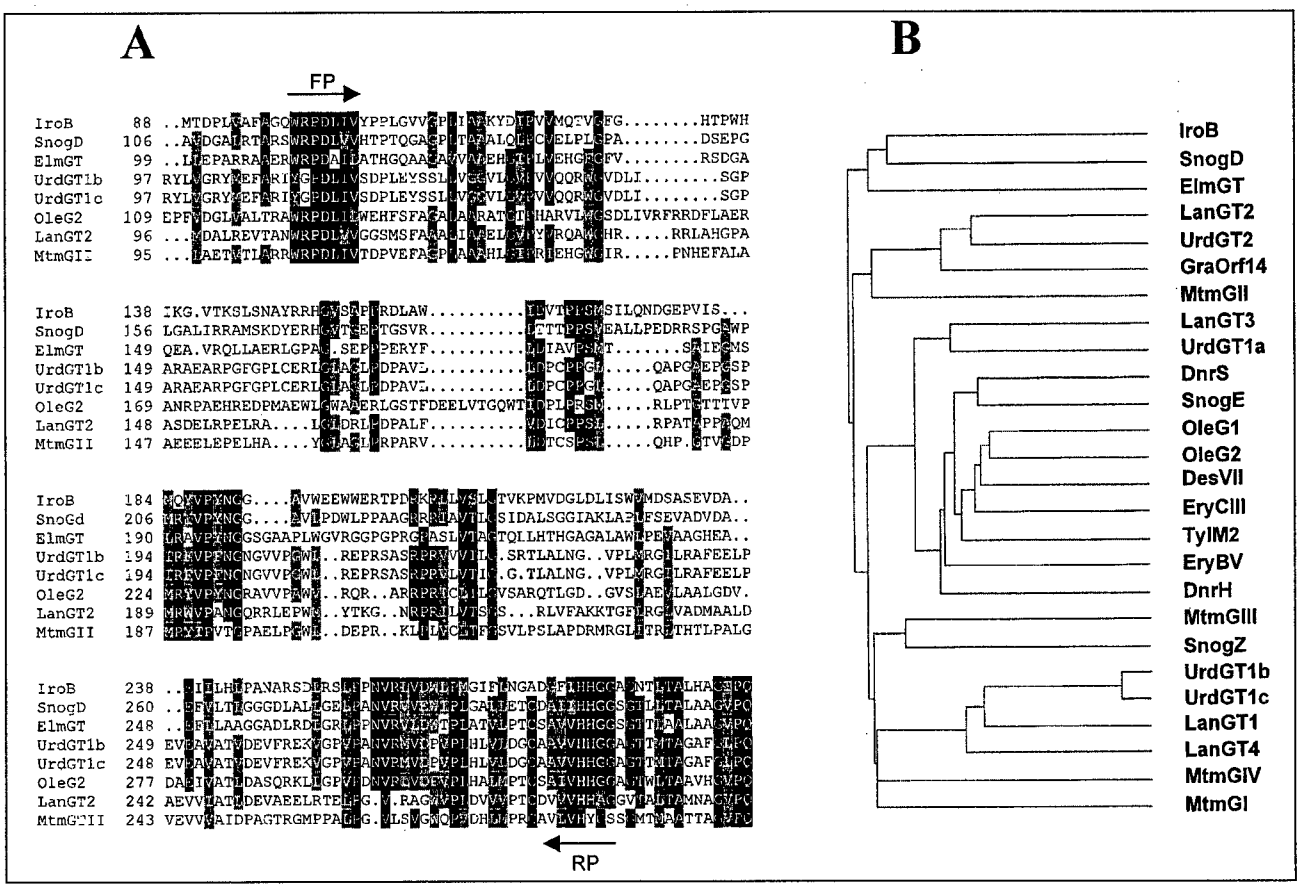

Fig. 3. (A) Alignment of the deduced amino acid sequences of different glycosyltransferases around the conserved regions chosen for designing the two oligoprimers for PCR amplification. FP and RP indicate the amino acid regions selected for the forward and reverse primer, respectively. IroB, glycosyltransferase from *S. typhi* [24]; SnogD, glycosyltransferase from *S. nogalater* [23]; UrdGT1b and UrdGT1c, glycosyltransferases from *S. fradiae* [3]; OleG2, glycosyltransferase from *S. antibioticus* [8]; LanGT2, glycosyltransferase from *S. cyanogenus* [11]; MtmGII, glycosyltransferase from *S. argillaceus* [7]. (B) Dendrogram showing the similarities among different glycosyltransferases: all glycosyltransferases shown are involved in antibiotic biosynthesis in producer organisms, with the exception of IroB.

Table 2
Biotransformation experiments to assay in vivo the activity of the ElmG glycosyltransferase in *S. albus* using different constructs directing the biosynthesis of several 6-deoxysugars

Construct	Genes	Sugar synthesized	Compound generated
pRHAM	▶ oleL oleS oleE oleU	L-rhamnose	RHA-TCM
pOLV	oleW oleV oleL oleS oleE oleU	L-olivose	OLV-TCM
pOLE	oleW oleV oleL oleS oleE oleU ▶ oleY	L-oleandrose	OLV-TCM

The black triangle indicates the position and orientation of the erythromycin resistance promoter.

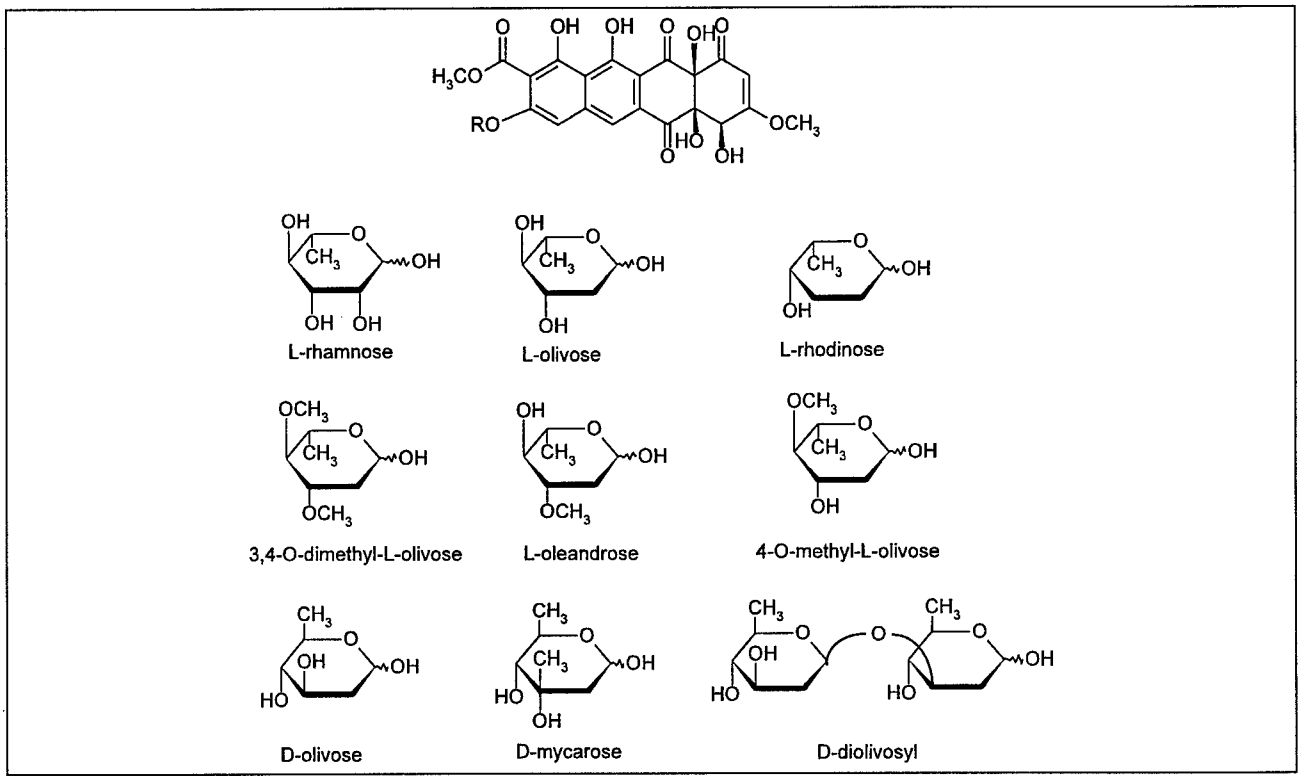

Fig. 4. Structures of 8-DMTC and 6-DOH (substituted at R) forming part of novel glycosylated tetracenomycin derivatives generated with the participation of the ElmGT glycosyltransferase.

rhamnose, dTDP-L-olivose and dTDP-L-oleandrose, respectively [20,25]. Transformants were selected for resistance to thiostrepton. The double recombinant strains harboring the *elmGT* gene integrated into the chromosome and the replicative plasmids directing the biosynthesis of different 6-DOH were used for biotransformation experiments. They were incubated for 24 h in liquid medium and then 8-DMTC was added (100 µg/ml final concentration). After 2 days of further incubation, the bioconversion of 8-DMTC to glycosylated derivatives was monitored (thin layer chromatography (TLC) and HPLC). In the presence of pRHAM, bioconversion of 8-DMTC was a very efficient process. Since it was previously shown [20] that pRHAM directs the biosynthesis of dTDP-L-rhamnose, it was assumed that the bioconverted product was L-rhamnosyl-tetracenomycin C. The product was purified and nuclear magnetic resonance analysis confirmed this presumption. Strains harboring either pOLV or pOLE gave a good bioconversion of 8-DMTC into the same compound, L-olivosyl-tetracenomycin C (LOLV-TCM), as it was confirmed by running the bioconversion products in parallel with pure LOLV-TCM as standard. L-Olivose has been proposed to be an intermediate in L-oleandrose biosynthesis in *S. antibioticus* [25]. These experiments confirm that ElmGT can recognize L-2,6-deoxysugars (i.e. L-olivose) in addition to its natural sugar substrate, an L-6-deoxysugar like L-rhamnose. The *elmGT* gene was also expressed (in pGB15) in the *S. argillaceus* mutant M3ΔMG in order to verify its capability to transfer D-sugars. Transformants were selected by their resistance to thiostrepton and used in biotransformation experiments with 8-DMTC. Formation of the monoglycosylated tetracenomycin derivatives (DOLV-TCM and DMYC-TCM) was observed but the presence of the diglycosylated derivative (DDIOLV-TCM) was not detected, suggesting the involvement of some mithramycin glycosyltransferases in the formation of the disaccharide derivative.

2.4. Formation of DDIOLV-TCM requires the cooperation of two mithramycin glycosyltransferases and the elloramycin glycosyltransferase, ElmGT

As shown above, the diglycosylated tetracenomycin DDIOLV-TCMC was not produced in *S. argillaceus* mutant M3ΔMG in which all the mithramycin glycosyltransferases were absent. This cannot be explained by the absence of an intracellular pool of dTDP-D-olivose in this mutant since the monoglycosylated derivative DOLV-TCMC was still produced. This raised an interesting question about how the diolivosyl disaccharide was formed and transferred. From the above shown results, it can be concluded that ElmGT plus some mithramycin glycosyltransferases are required for the biosynthesis of DDIOLV-TCM. Mithramycin contains a diolivosyl disaccharide attached to the C-6 position of the mithramycinone aglycon. It has been proposed that during mithramycin biosynthesis

this disaccharide is not formed by a sequential addition of the two D-olivose moieties but rather a diolivosyl disaccharide is firstly formed and then transferred [7]. The *mtmGI* and *mtmGII* gene products would be responsible for this synthesis and incorporation, although it is not yet clear which one was responsible for the formation of the disaccharide and its transfer [7]. Therefore, we thought that the biosynthesis of DDIOLV-TCM should require either one or both MtmGI and MtmGII glycosyltransferases for the formation of the diolivosyl disaccharide and ElmGT to transfer the disaccharide. To test this hypothesis, 16F4 was used to transform protoplasts of two *S. argillaceus* mutants (M3G1 and M3G2 mutants), in which either *mtmGI* or *mtmGII* genes were independently inactivated, respectively [7]. Analysis of the products synthesized by these recombinant strains showed that only the monoglycosylated tetracenomycins were produced, suggesting that the presence of both mithramycin glycosyltransferases was required for the formation of DDIOLV-TCM. Further confirmation was obtained when *S. argillaceus* M3ΔMG containing pGB15 (and therefore expressing *elmGT*) was independently transformed with pEM4A (control), pEPG1 (expressing *mtmGI*), pEPG2 (expressing *mtmGII*) or pEPG12 (expressing both *mtmGI* and *mtmGII*). Selected clones from these transformations were then incubated in the presence of 8-DMTC. Analysis of the biotransformation products after 2 days of incubation showed that no DDIOLV-TCM formation was observed with clones individually expressing *mtmGI* or *mtmGII* but DDIOLV-TCM was detected in the culture supernatant of clones that simultaneously expressed both mithramycin glycosyltransferases. From these results three main conclusions can be drawn: (i) addition of the diolivosyl disaccharide does not occur by sequential incorporation of the two sugars but rather by the direct transfer of the disaccharide; (ii) the MtmGI and MtmGII glycosyltransferases are responsible for the formation of the diolivosyl disaccharide and (iii) the disaccharide is then transferred by ElmGT to 8-DMTC.

2.5. Sugar flexible glycosyltransferases for generating new bioactive compounds

For the generation of novel glycosylated compounds by combinatorial biosynthesis, a certain degree of substrate flexibility of the glycosyltransferases is required. Recent studies by different laboratories have shown that some glycosyltransferases from different antibiotic biosynthetic pathways have relaxed substrate specificity. Glycosyltransferases involved in sugar transfer during the biosynthesis of glycopeptide antibiotics have been shown to transfer sugars to different, but structurally related, heptapeptides [26]. In addition, some macrolide glycosyltransferases can transfer different sugars into an aglycon [10,25,27–29]. The ElmGT we reported here adds a new and important example of sugar flexibility to antibiotic glycosyltransferases. ElmGT apparently shows a broader flexibility for the sugar substrate than any other glycosyltransferase from antibiotic pathways so far described. ElmGT is capable of transferring to its natural substrate aglycon (8-DMTC) different L-DOHs as its natural sugar substrate, L-rhamnose (6-DOH), L-olivose (2,6-DOH) and L-rhodinose (2,3,6-DOH) (Fig. 4). In addition, it can also transfer some D-sugars as D-olivose and D-mycarose (2,6-DOHs) and it can even transfer a D-olivose disaccharide. Interestingly, for the biosynthesis of this disaccharide the joint action of two mithramycin glycosyltransferases (MtmGI and MtmGII) is required. As far as we know this is the first report on antibiotic biosynthetic pathways in which a collaborative action of two glycosylating enzymes for the formation of a disaccharide has been stated.

3. Significance

The generation of novel bioactive compounds with an altered glycosylation pattern requires substrate flexibility of the glycosyltransferases. The rhamnosyl glycosyltransferase ElmGT from *S. olivaceus*, and other flexible glycosyltransferases, constitute powerful tools for producing novel hybrid glycoside antibiotics through the incorporation of different sugar moieties into the corresponding aglycons. This will require an understanding of which amino acid residues/regions are involved in the recognition of the cofactor (dTDP-activated 6-DOH) and the substrate (aglycon) to act on specific amino acid residues in order to improve sugar flexibility.

4. Materials and methods

4.1. Bacterial strains, culture conditions and vectors

S. olivaceus Tü2353, the elloramycin producer, was used as the source of chromosomal DNA. The following wild type *Streptomyces* strains were used as transformation hosts: *S. albus G*, *S. lividans* TK21 and *S. argillaceus* ATCC 12956. In addition, several mithramycin non-producing mutants were also used as transformation hosts: *S. argillaceus* M3ΔMG [21] and *S. argillaceus* M3G1 and M3G2 [7]. Growth was carried out on trypticase soya broth (TSB; Oxoid) or R5A medium [7]. For sporulation, growth was for 7 days at 30°C on agar plates containing A medium [7]. Protoplast formation and transformation were carried out using standard procedures [30] with some minor modifications for *S. argillaceus* [31]. *Escherichia coli* XL1-Blue [32] was used as a host for subcloning and was grown at 37°C in TSB medium (Oxoid). The bifunctional (*Streptomyces–E. coli*) replicative plasmid vectors pIAGO [25], pEM4A (this paper) and the integrative vector pKC796 [33] were used for gene expression in *Streptomyces*. pEM4A is a pEM4 derivative [34] containing the apramycin resistance cassette subcloned as a blunt-ended *Hin*dIII–*Bam*HI fragment from pEFBA [35] into the unique *Eco*RV site located

within the thiostrepton resistance gene. Consequently, pEM4A confers resistance to apramycin and not to thiostrepton. When plasmid-containing clones were grown, the medium was supplemented with the appropriate antibiotics: 5 or 50 μg/ml thiostrepton for liquid or solid cultures, respectively, 100 μg/ml for ampicillin, 2.5 or 25 μg/ml apramycin for liquid or solid cultures, respectively, or 20 μg/ml tobramycin.

4.2. DNA manipulation and sequencing

Plasmid DNA preparations, restriction endonuclease digestions, alkaline phosphatase treatments, ligations and other DNA manipulations were performed according to standard procedures for *E. coli* [36] and for *Streptomyces* [30]. Sequencing was performed on double-stranded DNA templates in pUC18 by using the dideoxynucleotide chain-termination method [37] and the Cy5 AutoCycle Sequencing Kit (Pharmacia Biotech). Both DNA strands were sequenced with primers supplied in the kits or with internal oligoprimers (17-mer) using an ALF-express automatic DNA sequencer (Pharmacia). Computer-aided data base searching and sequence analyses were carried out using the University of Wisconsin Genetics Computer Group programs package (UWGCG) [22] and the BLAST program [38].

4.3. PCR amplification

Degenerated oligoprimers were designed to try to locate the presence of the *elmGT* glycosyltransferase gene in cosmid 16F4. They were derived from conserved amino acid sequences present in a number of different antibiotic glycosyltransferases (see Fig. 3A). These oligoprimers were used to attempt PCR amplification using as template cosmid 16F4 DNA or subclones derived from it. The two oligoprimers were: forward primer (5′) CG (C,G) T (A,T) CGT (C,G) CC (C,G) T (A,T) CAACGG (3′) and reverse primer (5′) GT (C,G) CC (C,G) (C,G) C (C,G) CC (C,G) (C,G) CGTGGTG (3′). The PCR reaction conditions were as follows: 100 ng of template DNA was mixed with 30 pmol of each primer and 2 U of Taq DNA polymerase (Boehringer) in a total reaction volume of 100 μl containing 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 2.5% DMSO (Merck) and 10% glycerin. This reaction mix was overlayed with 50 μl of mineral oil (Sigma) and the polymerization reaction was performed in a thermocycler (MinyCycler, MJ Research) under the following conditions: an initial denaturation of 5 min at 96°C; 35 cycles of 90 s at 95°C, 30 s at 60°C and 90 s at 72°C; after the 35 cycles, an extra extension step of 10 min at 72°C was added, followed by cooling at 4°C at the end of this program. The PCR product was used as DNA probe for Southern hybridization.

4.4. Gene expression

For the expression of *elmGT* in *Streptomyces*, the gene was amplified by PCR. The following oligoprimers were used: (5′) ATCGTCTAGAGGCTGCGGGAAGCGACCTGACATG (3′) for the 5′-end of the gene and (5′) ATCGAAGCTTGTCGCAGCGGGCCGTGAGGTCA (3′) for the 3′-end of the gene (restriction sites for *Xba*I and *Hin*dIII sites were included in the oligoprimers, respectively, to facilitate subcloning and are underlined). PCR reaction conditions were as follows: 100 ng of template DNA was mixed with 30 pmol of each primer and 2 U of Vent DNA Polymerase (New England Biolabs) in a total reaction volume of 50 μl containing 2 mM of each dNTP and 10% DMSO (Merck). This reaction mix was overlayed with 50 μl of mineral oil (Sigma) and the polymerization reaction was performed in a thermocycler (MinyCycler, MJ Research) under the following conditions: an initial denaturation of 3 min at 98°C; 30 cycles of 30 s at 95°C, 60 s at 68°C and 90 s at 72°C; after the 30 cycles, an extra extension step of 5 min at 72°C was added, followed by cooling at 4°C at the end of this program. The PCR product was digested with *Xba*I and *Hin*dIII and subcloned into the same sites of pUC18. The fragment was then rescued as an *Xba*I–*Hin*dIII DNA fragment and subcloned into the same sites of pIAGO, immediately downstream of the erythromycin resistance promoter (*ermE*p), generating pGB15. From this construct, a 1.4 kb *Bgl*II fragment, containing both the *ermE*p and *elmGT* gene, was rescued and subcloned into the unique *Bam*HI site of pKC796, generating pGB16.

For expression of the different mithramycin glycosyltransferases, the following constructs were made: (i) *mtmGI*: a 2.5 kb *Eco*RI–*Hin*dIII fragment from pIAGOG1 [12], that contained the *mtmGI* gene under the control of the *ermEp*, was subcloned in the same sites of pEM4A, generating pEPG1. (ii) *mtmGII*: a 2.3 kb *Eco*RI–*Hin*dIII fragment from pIAGOG2 [12], that contained the *mtmGII* gene under the control of the *ermEp* promoter, was subcloned in the same sites of pEM4A, generating pEPG2. (iii) *mtmGI+mtmGII*: a 5.1 kb *Hin*dIII–*Xba*I fragment from pM12G12 [E. Fernández, unpublished results], that contained the methyltransferase genes *mtmMI* and *mtmMII* [13] and the glycosyltransferase genes *mtmGI* and *mtmGII* [12], was subcloned into the same sites of pEM4A, generating pEPG12. In this construct both glycosyltransferase genes are under the control of their own promoters.

4.5. Preparation of 8-DMTC

Spores of strain *S. argillaceus* 16F4 were inoculated in TSB medium supplemented with 2.5 μg/ml apramycin. After 24 h of incubation at 30°C and 250 rpm, this culture was used to inoculate (at 2.5%, v/v) eight 2 l Erlenmeyer flasks containing 400 ml of R5A medium [7] also containing apramycin. After further incubation for 5 days under the above conditions, the cultures were centrifuged, filtered and extracted as previously described [7]. 8-DMTC was subsequently purified by reverse-phase preparative HPLC. The mobile phase used for elution in isocratic conditions was a mixture of acetonitrile and 0.1% trifluoroacetic acid in water (30:70). The peak corresponding to 8-DMTC was collected, dried in vacuo and weighted, resulting in 638 mg of pure compound as determined by analytical HPLC.

4.6. Biotransformation

For biotransformation experiments, spores of the appropriate

S. albus recombinant strains were used to inoculate 5 ml of TSB liquid medium (containing the selection antibiotic) for 24 h at 30°C and 250 rpm. Then, 100 μl of this culture was used to inoculate 5 ml R5A liquid medium. After 24 h of incubation, 8-DMTC was added at 100 μg/ml final concentration and the cultures further incubated. At 24 h intervals, 900 μl samples were removed and mixed with 300 μl ethyl acetate. After centrifugation to separate the aqueous and organic phases, the organic phase was removed, evaporated under vacuo and the residue resuspended in a small volume of methanol.

4.7. TLC and HPLC analyses

For TLC analysis, 1–2 μl samples were applied onto pre-coated silica gel 60 F_{254} plates and chromatography developed using dichloromethane/methanol (90/10, v/v) as a solvent. Detection was carried out by UV light absorption. HPLC analyses of ethyl acetate extracts were performed as previously described [35].

Acknowledgements

We would like to thank C.R. Hutchinson for providing us pWHM1026Δ*tcm*O. This work was supported by grants of the European Community to J.R., A.B. and J.A.S. (BIO4-CT96-0068), the Spanish Ministry of Education and Science to J.A.S. through the 'Plan Nacional en Biotecnologia' (BIO97-0771), and the South Carolina Commission of Higher Education and the US Department of Defense to J.R.

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Digitoxosyltetracenomycin C and Glucosyltetracenomycin C, Two Novel Elloramycin Analogs Obtained by Exploring the Sugar Donor Substrate Specificity of Glycosyltransferase ElmGT

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Our explorations of glycosyltransferase ElmGT from *Streptomyces olivaceus* Tü 2353, which showed an interesting flexibility regarding its sugar donor substrate, were extended towards various previously unexplored sugar co-substrates. The studies revealed that ElmGT, which normally transfers L-rhamnose to 8-demethyltetracenomycin C as a crucial biosynthetic step in the elloramycin biosynthesis, is also able to process an activated non-deoxygenated sugar, NDP-D-glucose as well as NDP-L-digitoxose, which is the first example of an NDP-L-sugar co-substrate of ElmGT possessing an axial 3-OH group. The structures of the resulting novel elloramycin analogs of these experiments, 8-demethyl-8-L-digitoxosyl-tetracenomycin C and 8-demethyl-8-D-glucosyl-tetracenomycin C, were elucidated mainly by ^{1}H and ^{13}C NMR spectroscopy and by mass spectrometry.

Glycosyltransfer reactions are perhaps the most important biotranformations on Earth, at least on quantitative terms, considering the biosynthesis of bulky biomass such as cellulose or chitin, energy storage polymers (starch), and molecules responsible for bio-information nucleic acids.[1,2] Glycosyltransferases are also appealing enzymes for drug discovery programs applying a combinatorial biosynthesis approach. First, glycosyltransferases are responsible for the attachment of sugar moieties, often deoxysugars, which add important features to the shape and the stereo-electronic properties of a molecule and often play an essential role for the biological activity of many natural product drugs.[3-6] Second, it has been shown recently that many glycosyltransferases have naturally an unexpected inherent substrate flexibility either towards their acceptor substrate (usually alcohols or phenols) or regarding their (deoxy)sugar donor co-substrates, sometimes even to both.[7-10] Thus, glycosyltransferases are and will become more and more important tools for combinatorial biosynthetic approaches.[3,11]

ElmGT[12] is a glycosyltransferase (GT), which connects L-rhamnose with 8-demethyltetracenomycin C (**1**) to form the glycosylated late intermediate **2** of the elloramycin (**3**) biosynthesis, which then only needs three more *O*-methylation steps at the deoxysugar as well as one at the aglycone moiety (12a-position) to finish the biosynthesis of the antitumor agent elloramycin (**3**, Figure 1).[13] The elloramycins[13-17] and tetracenomycins[18-23] form a distinct group of tetracyclic aromatic polyketides, which is remotely related to the anthracyclines and tetracyclines. *elmGT* is a gene coding for a glycosyltransferase in the elloramycin biosynthetic gene cluster.[12] This gene was isolated from cosmid 16F4 that contains a 25 kb DNA region of this cluster including all genes

necessary for the formation of the polyketide aglycone moiety of elloramycin as well as the methyltransferases necessary to methylate all OH groups of the sugar moiety.[12-14,24] Thus, from the entire elloramycin biosynthesis gene cluster, only the genes encoding the biosynthesis of L-rhamnose are missing in cosmid 16F4. The tremendously relaxed specificity of ElmGT towards its NDP-sugar donor substrate became obvious after heterologous expression of cosmid 16F4 into the urdamycin producer *Streptomyces fradiae*[25,26] and into the mithramycin producer *Streptomyces argillaceus*.[25] ElmGT was able to glycosylate its acceptor substrate 8-demethyltetracenomycin C (**1**) with L-rhodinose, D-olivose, D-mycarose, and even a disaccharide consisting of two D-olivose moieties (Figure 2). All the NDP-sugar co-substrates were provided by the host strains, while it was evident (and later confirmed by follow-up experiments, in which glycosyltransferases of the host strains did not exist or were inactivated) that ElmGT, and not one of the host GTs was responsible for the linkage of the various sugar building blocks.[12] In addition, the substrate specificity of ElmGT was further explored through constructs, in which selected genes of the sugar building block of oleandomycin, oleandrose, were combined with cosmid16F4 in a neutral host.[13] This work showed that ElmGT can process NDP-L-rhamnose and NDP-L-olivose (Figure 2). It was also shown that ElmGT could not transfer 4-keto-L-rhamnose, which exemplified the first 'shortcoming' of ElmGT in respect of its broad substrate specificity towards its NDP-sugar donor substrate.

In this communication we report further investigations of ElmGT towards its NDP-sugar donor substrate. We were interested whether ElmGT can handle (i) L-sugars with an axial 3-OH group and (ii) non-deoxysugars. To test these alternatives, the following

general approach was used. *Streptomyces lividans* served as host strain, into which two plasmids were heterologously expressed, namely (i) cosmid 16F4, which contains all genes necessary for the biosynthesis of 8-demethyltetracenomycin C (1), the GT encoding gene *elmGT*, and three methyltransferase encoding genes, and (ii) different plasmid constructs directing the biosynthesis of NDP-L-sugars. Upon cultivation of the different recombinant strains, formation of novel glycosylated derivatives was analyzed by HPLC and, if appropriate, compounds isolated and structures elucidated.

To assay the first alternative (i.e. L-sugars with an axial 3-OH group), we used pLNB43. This is a pLN2[27] derivative which contains all L-oleandrose genes from the oleandomycin producer *Streptomyces antibioticus*, except *oleY* (encoding the *O*-methyltransferase),[28] which was substituted by *eryBIII* (encoding the 3C-methyltransferase of the L-cladinose building block of eythromycin A), and *oleU* (encoding the 4-ketoreductase), which was replaced by the analogous gene *eryBIV* (encoding the 4-ketoreductase of the L-cladinose biosynthesis) of the erythromycin producer *Saccharopolyspora erythraea*. We anticipated the following possibilities for the outcome of this experiment:

a) The 'sugar plasmid' leads to the construction of activated L-mycarose, thus the experiment should yield L-mycarosyltetracenomycin C.

b) The erythromycin C-methyltransferase EryBIII fails, and the activated sugar encoded by plasmid pLNB43 yields either NDP-L-digitoxose or NDP-L-olivose or both, depending how the stereochemistry for the 3-position is controlled (does EryBIII contribute at least to the stereochemical outcome or not at all, does EryBIV influence the

3-stereochemistry?). Thus the experiment should yield L-olivosyltetracenomycin C (a known product from a previous experiment) or L-digitoxosyltetracenomycin C, or both. c) The erythromycin ketoreductase EryBIV fails, and plasmid pLNB43 directs the biosynthesis of a 4-ketosugar, either with an equatorial or an axial OH group in 3-position, either with or without the 3-*C*-methyl group (depending on EryBIII). Thus the result of the experiment may also be 4-keto-L-olivosyltetracenomycin C, 4-keto-L-mycarosyltetracenomycin C or 4-keto-L-digitoxosyltetracenomycin C.

The product spectrum of *S. lividans* (16F4, pLNB43) yielded one major product and two minor products. The two latter were known products and readily identified by HPLC retention time and UV spectrum as 8-demethyl-8-L-olivosyltetracenomycin C (**5**) and 8-demethyl-8-L-oleandrosyltetracenomycin C (**6**, Figure 3). The main product was isolated and its elucidated structure turned out to be 8-demethyl-8-L-digitoxosyltetracenomycin C (**4**, Figure 3). This was evident from the HRMS, showing molecular ions at *m/z* 611.1359 ($M+Na^+$, 100%) and 589.1550 ($M+H^+$, 10%) which is consistent with a molecular formula of $C_{28}H_{28}O_{14}$, and from the NMR data. The ^{13}C NMR shows the signals expected for the aglycone moiety (8-demethyltetracenomycin C), and six extra signals consistent with one deoxyhexose moiety (table 1). The analysis of the ^1H NMR regarding this deoxysugar unit revealed that the sugar is α-glycosidically linked to a phenolic oxygen atom (δ_H of 1'-H: 5.97 d, J = 3.5 Hz), and that it is a 2,6-dideoxysugar, with an equatorial 4-OH and an axial 3-OH group. This stereochemistry can be concluded from the signal patterns of 2'-H_a (δ_H 2.20 ddd, J = 15, 3.5, 3.5 Hz) and 2'-H_e (δ_H 2.28 ddd, J = 15, 3.0, 1.0 Hz), which show both only small couplings with 3'-H (after D_2O-exchange: δ_H 4.04 ddd, J = 3.5, 3.0, 3.0 Hz). The 4'-H signal (after D_2O-exchange: δ_H 3.27 dd, J = 9.5, 3.0

Hz) shows one small (to 3'-H) and one large coupling (to 5'-H), thus, indicating its axial position. The HMBC spectrum reveals a coupling between 1'-H and C-8 proving the attachment of the sugar moiety to the phenolic 8-O-atom.

In respect of non-deoxysugars, we attempted to search for minor compounds, since at least activated D-glucose is ubiquitous, and thus is also produced by *S. lividans*. If ElmGT is able to handle activated non-deoxysugars then D-glucosyltetracenomycin C might be formed as a minor side product of the experiments. When analyzing extracts from different recombinant strains harboring cosmid 16F4 and different "sugar plasmids", we frequently noticed that there was a minor HPLC peak in ethyl acetate extracts of these strains. We also found that, in some recombinant strains, the size of this peak greatly increased when the culture supernatants were directly analyzed (without organic solvent extraction) by HPLC. Its yield was particularly high in *S. lividans* containing cosmid 16F4 and plasmid pRHAM[29] or derivatives.[24] This new product was isolated and elucidated as 8-demethyl-8-D-glucosyltetracenomycin C (**7**, Figure 3). The HREIMS shows molecular ions at *m/z* 643.1298 (100%, $M+Na^+$) and at *m/z* 621.1483 (30%, $M+H^+$), both in agreement with a molecular formula of $C_{28}H_{28}O_{16}$. The NMR spectra indicate besides the known aglycone 8-demethyltetracenomycin C the presence of an additional sugar moiety, which is, considering the chemical shifts of its six ^{13}C NMR signals and its ^1H NMR signals, not a deoxysugar. The coupling constants (all signals have only large coupling constants) of the sugar show its β-glycosidic linkage, and are in agreement with the glucose stereochemistry. A cross signal of the HMBC spectrum between 1'-H and C-8 prove the linkage of this D-glucose moiety to the 8-position, as expected.

The results described here prove for the first time that ElmGT is capable to process (i) the non-deoxygenated activated sugar NDP-D-glucose, and (ii) NDP-L-digitoxose, i.e. an activated L-sugar, whose 3-OH is in an axial position. Furthermore, no 4-ketosugar derivative of 8-demethyltetracenomycin could be found. Thus, ketoreductase EryBIV, which normally reduces NDP-4-keto-L-mycarose, was able to act on a substrate lacking the equatorial 3-*C*-methyl group. Since the natural substrate of EryBIV also has an axial 3-OH group, it is likely that EryBIV is responsible for this step, although it cannot be ruled out that a 4-ketoreductase from *S. lividans* performed this reduction.[30] It is unlikely though that NDP-4-keto-L-digitoxose or NDP-4-keto-L-olivose were present, so that no further conclusions can be drawn regarding ElmGT's substrate specificity towards 4-ketosugars. It is somewhat surprising that EryBIII was unable to *C*-methylate NDP-4-keto-L-olivose, since this activated L-sugar (or its 3-epimer [31]) is discussed as its natural substrate.[30,32,33] However, an NDP-D-sugar substrate, namely NDP-4-keto-D-digitoxose (= 2,6-dideoxy-D-glycero-4-hexulose),[34,35] was recently established for an analog of this enzyme, TylC3, which catalyzes exactly the same *C*-methylation step for the formation of the NDP-L-mycarose building block within the tylosin biosynthesis. These findings also suggest an alternative sequence of events, i.e. 2-deoxygenation and 3-*C*-methylation prior to 5-epimerization, for the NDP-L-mycarose biosynthesis in *S. erythraea* (see Figure 4, dotted box). Our results here support this view, although we cannot completely rule out that *eryBIII* is not working in our plasmid construct (e.g., because of a PCR amplification mistake). Since our experiment seems to render EryBIII unnecessary for the formation of NDP-L-digitoxose, we also used plasmid pLNBIV,[27] a pLN2 derivative containing *eryBIV* but lacking *eryBIII*. This plasmid was heterologously expressed into *S. lividans*

together with cosmid 16F4, and the results were essentially the same as in the experiment described above: production of mainly 8-demethyl-8-L-digitoxosyltetracenomycin C (**4**) along with smaller amounts of 8-demethyl-8-L-olivosyltetracenomycin C (**5**). In addition, the production of 8-demethyl-8-L-oleandrosyltetracenomycin C (**6**) was also observed as a consequence of the participation of the *elmMII O*-methyltransferase encoding gene in cosmid 16F4. These follow-up experiments proved that EryBIII does not contribute to the generation of L-digitoxosyltetracenomycin C (**4**), which probably means that the stereochemistry in 3'-position of this metabolite is controlled solely by ketoreductase EryBIV. It is likely that both NDP-sugars, NDP-4-keto-L-olivose and NDP-4-keto-L-digitoxose, are in an equilibrium due to tautomerism (Figure 4, solid box). Since the latter is the better substrate for EryBIV due to its axial 3-OH group (like in its natural suggested substrate NDP-4-keto-L-mycarose), digitoxosyltetracenomycin C (**4**) is always the major product of these experiments involving the *eryBIV* gene, while L-olivosyltetracenomycin C (**5**) is always the minor product.

Experimental Section

General Experimental Procedures. CD spectra were recorded on a AVIV circular dichroism spectropolarimeter (Model 60DS) in a 1 cm cell. UV spectra were recorded on a Beckman DU 650 spectrophotometer, IR spectra were obtained from pure samples on KCl disks in a Mattson Genesis II FT FT-IR spectrometer, and NMR spectra were recorded in d_6-acetone or CD_3OD on a Varian Inova 400 NMR instrument at 400 MHz for ^1H and at 100.6 MHz for ^{13}C, using 1D spectra and 2D homo- and heteronuclear

correlation experiments (^{1}H, ^{13}C, DEPT, H,H-COSY, HSQC, and HMBC). Electron-impact mass spectrometry (EIMS) was carried out using a VG 70SQ double-focusing magnetic sector mass spectrometer with sample introduction by direct exposure probe and ionization energy of 70eV. HPLC was performed on a Waters HPLC system (Delta 600, M32 add-on single system, with a photodiode array detector model 996), using the columns and guard columns described below.

Bacterial Strains and Plasmids.

Streptomyces lividans TK21 was used as transformation host. Growth was carried out on trypticase soy broth (TSB; Oxoid) or R5A medium.[36] For sporulation, cells were grown for 7 days at 30 °C on agar plates containing A medium.[36] Plasmids pLN2 and pLNBIV were previously described.[27] pLNB43 is a pLNBIV derivative in which the *oleY* gene was removed from pLNBIV by digestion with *Nhe*I and *Pac*I and then replaced by the *eryBIII* gene using the same restriction sites. When antibiotic selection was required 2.5 μg/ml (liquid medium) or 25 μg/ml (solid medium) of thiostrepton or apramycin were used.

Cultivation and Fermentation.

Spores of *S. lividans* (16F4, pLNB43) were initially grown in TSB medium for 24 h at 30 °C and 250 rpm. This seed culture was used to inoculate (at 2.5%, v/v) seven 2 l Erlenmeyer flasks each containing 400 ml of R5A medium,[36] and incubated for 6 days under the above conditions. A seed culture of *S. lividans* (16F4, pRHAM) was prepared

as above, and used to inoculate (at 2.5%, v/v) three 2 l Erlenmeyer flasks each containing 400 ml of R5A medium, which were grown for 60 hours.

Isolation of the New Products 4 and 7. The cultures were centrifuged, the supernatants were filtered, and applied to a solid-phase extraction cartridge (Supelclean LC-18; 10 g; Supelco). The cartridge was eluted with methanol and water, using a linear gradient from 0 to 100% methanol in 60 min, at 10 mL/min, taking fractions every 5 min. Fractions containing the products **4** or **7**, as assessed by HPLC analysis, were dried in vacuo and re-dissolved in a small amount of methanol:dimethylsulfoxide (1:1). The resulting products were purified by preparative HPLC using a μBondapak C_{18} radial compression cartridge (PrepPak Cartridge; 25 by 100 mm; Waters). An isocratic elution with a mixture of methanol and water (4:6), at 10 mL/min, allowed the separation of compound **7** as a pure peak, which was collected and dried in vacuo; 21 mg 8-Demethyl-8-D-glucosyltetracenomycin C (**7**) were isolated as amorphous yellow solid. For the isolation of compound 4, the extracted material was chromatographed as above, but using acetonitrile and 0.1% trifluoroacetic acid in water (27:73) as a mobile phase. The purified compound was diluted 4-fold with water, applied to a solid phase extraction column (Sep-Pak Vac, Waters), washed with water to eliminate trifluoroacetic acid, and finally eluted with methanol and dried in vacuum; 27.8 mg 8-Demethyl-8-L-digitoxosyltetracenomycin C (**4**) were isolated as amorphous yellow solid.

Characterization of 8-Demethyl-8-L-digitoxosyltetracenomycin C (4). CD (MeOH) $\lambda_{extr.}$ (Θ^{20}) 383 (49200), 313 sh (6800), 289 (-11600), 260 (2700), 213 (12300)

nm: UV (MeOH) λ_{max} (ε) 502 (1900), 435 (12000), 411 (13600), 286 (32500), 244 sh (28900) nm; (MeOH-HCl) λ_{max} (ε) 406 (15700), 388 (14600), 286 (51300), 238 (33100), 209 (24000) nm; (MeOH-NaOH) λ_{max} (ε) 507 (3200), 440 (14700), 422 sh (13400), 260 (35800), 205 (93800) nm; IR ν_{max} (KCl) 3373, 2956, 2358, 1713, 1671, 1596, 1436, 1365, 1228, 1101, 1058, 960, 901, 832, 631 cm^{-1}; ^1H NMR, ^{13}C NMR, and observed COSY and HMBC couplings: see Table 1; HREIMS *m/z* 611 ($[M+Na]^+$, 611.1359, calcd for $C_{28}H_{28}O_{14}Na$, 611.1377, 100), 589 ($[M+H]^+$, 589.1550, calcd for $C_{28}H_{29}O_{14}$, 589.1557, 10).

Characterization of 8-Demethyl-8-D-glucosyltetracenomycin C (7). CD (MeOH) $\lambda_{extr.}$ (Θ^{20}) 381 (53100), 372 (42800), 358 (46500), 284 (-700), 212 (27300) nm; UV (MeOH) λ_{max} (ε) 505 (2000), 437 (9500), 417 (9300), 263 (25400), 208 (24200) nm; (MeOH-HCl) λ_{max} (ε) 405 (9200), 390 (8700), 285 (34700), 237 (24400), 208 (21000) nm; (MeOH-NaOH) λ_{max} (ε) 441 (10700), 261 (27300), 203 (116900) nm; IR ν_{max} (KCl) 3337, 2942, 2050, 1706, 1663, 1595, 1366, 1227, 1066, 981, 940, 786, 830, 636 cm^{-1}; ^1H NMR, ^{13}C NMR, and observed COSY and HMBC couplings: see Table 1; HREIMS *m/z* 643 ($[M+Na]^+$, 643.1298, calcd for $C_{28}H_{28}O_{16}Na$, 643.1275, 100), 621 ($[M+H]^+$, 621.1483, calcd for $C_{28}H_{29}O_{16}$, 621.1456, 30).

Acknowledgments. This work was partly supported by grants from the U. S. Department of Defense, awarded to J. R., and the Spanish Ministry of Science and

Technology (Plan Nacional en Biotecnología BIO2000-0274) and the European Community (QLRT-1999-00095), awarded to J. A. S.

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Captions to Figures

Figure 1 The glycosyltransfer step catalyzed by ElmGT within the elloramycin biosynthesis; **1**: 8-demethyltetracenomycin C, **2**: L-rhamnosyltetracenomycin C, **3**: elloramycin A

Figure 2 NDP-sugar donor substrate flexibility of ElmGT (the shown stereochemistry at C-1 of the sugar moieties refers to the products)

Figure 3 Products of the newly generated hybrid strains (see text): L-digitoxosyltetracenomycin C (**4**) and D-glucosyltetracenomycin C (**7**) are novel products, **5** and **6** were already previously described as products of other constructions

Figure 4 Biosynthetic pathways to NDP-L-olivose (in the oleandomycin producer *S. antibioticus*), and suggested pathways to NDP-L-mycarose in *S. erythraea* (dotted box) and to NDP-L-digitoxose (in *S. lividans* (16F4, pLNB43) or *S. lividans* (16F4, pLNBIV) (solid box).

Fig. 1

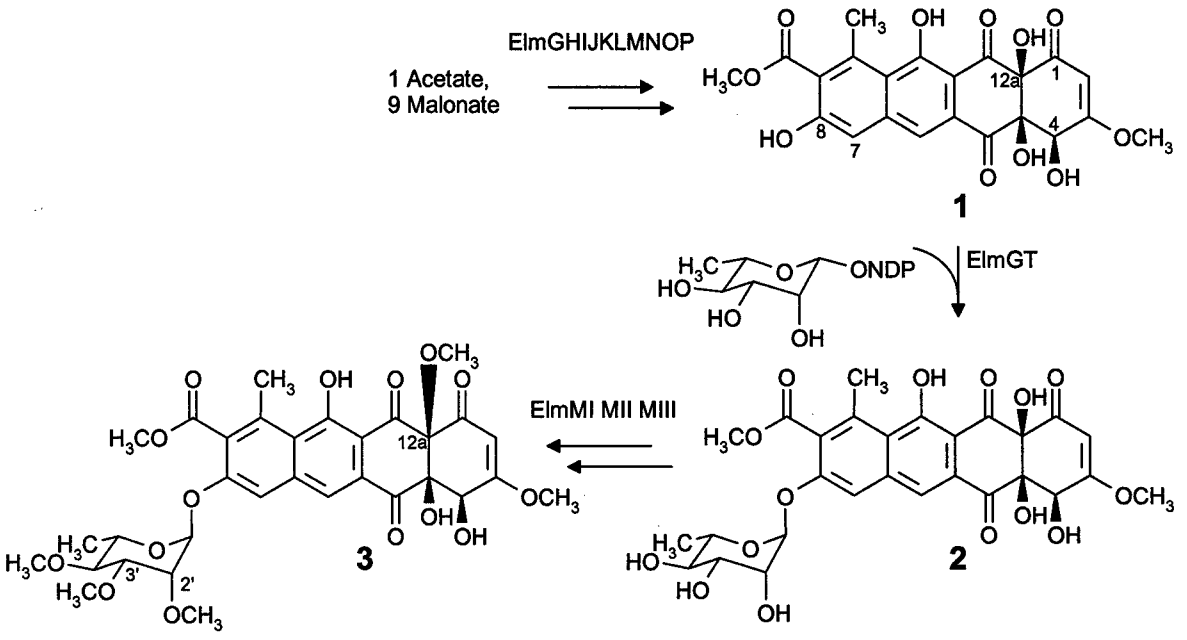

ElmGHIJKLMNOP
1 Acetate,
9 Malonate
1
ElmGT
ONDP
ElmMI MII MIII
2
3

Fig. 2

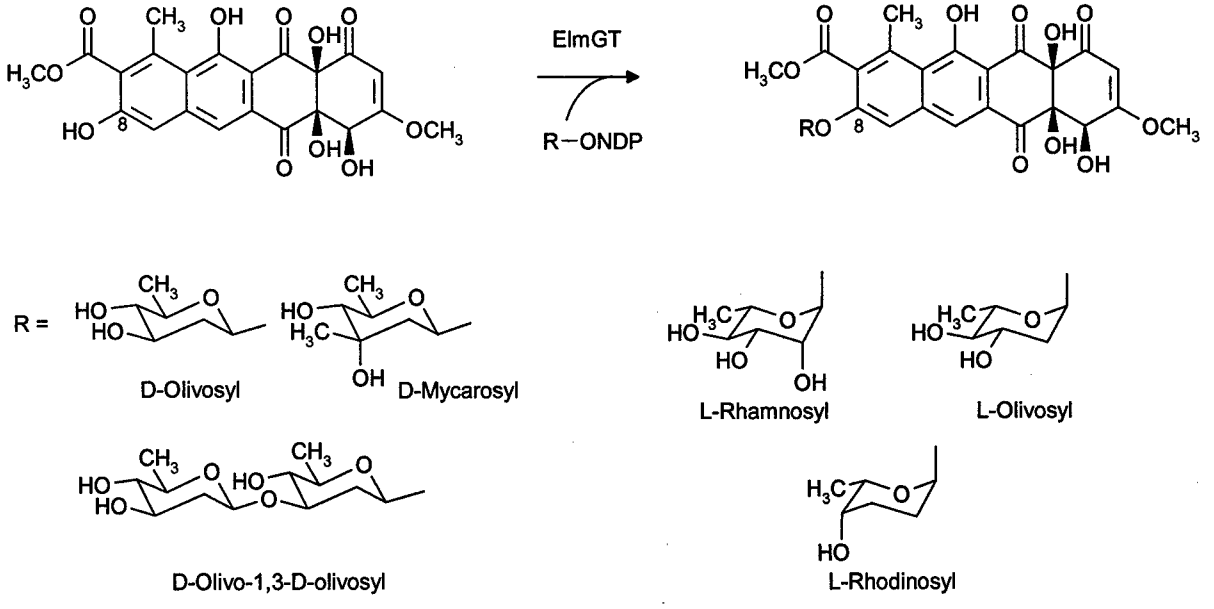
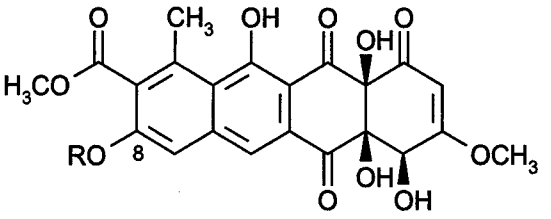
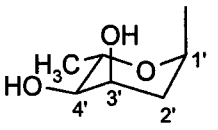
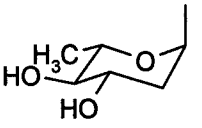
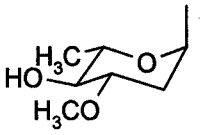

ElmGT
R—ONDP
R =
D-Olivosyl
D-Mycarosyl
L-Rhamnosyl
L-Olivosyl
D-Olivo-1,3-D-olivosyl
L-Rhodinosyl

Fig. 3



4, R = 

5, R = 

6, R = 

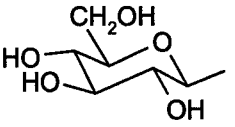
7, R = 

Fig. 4

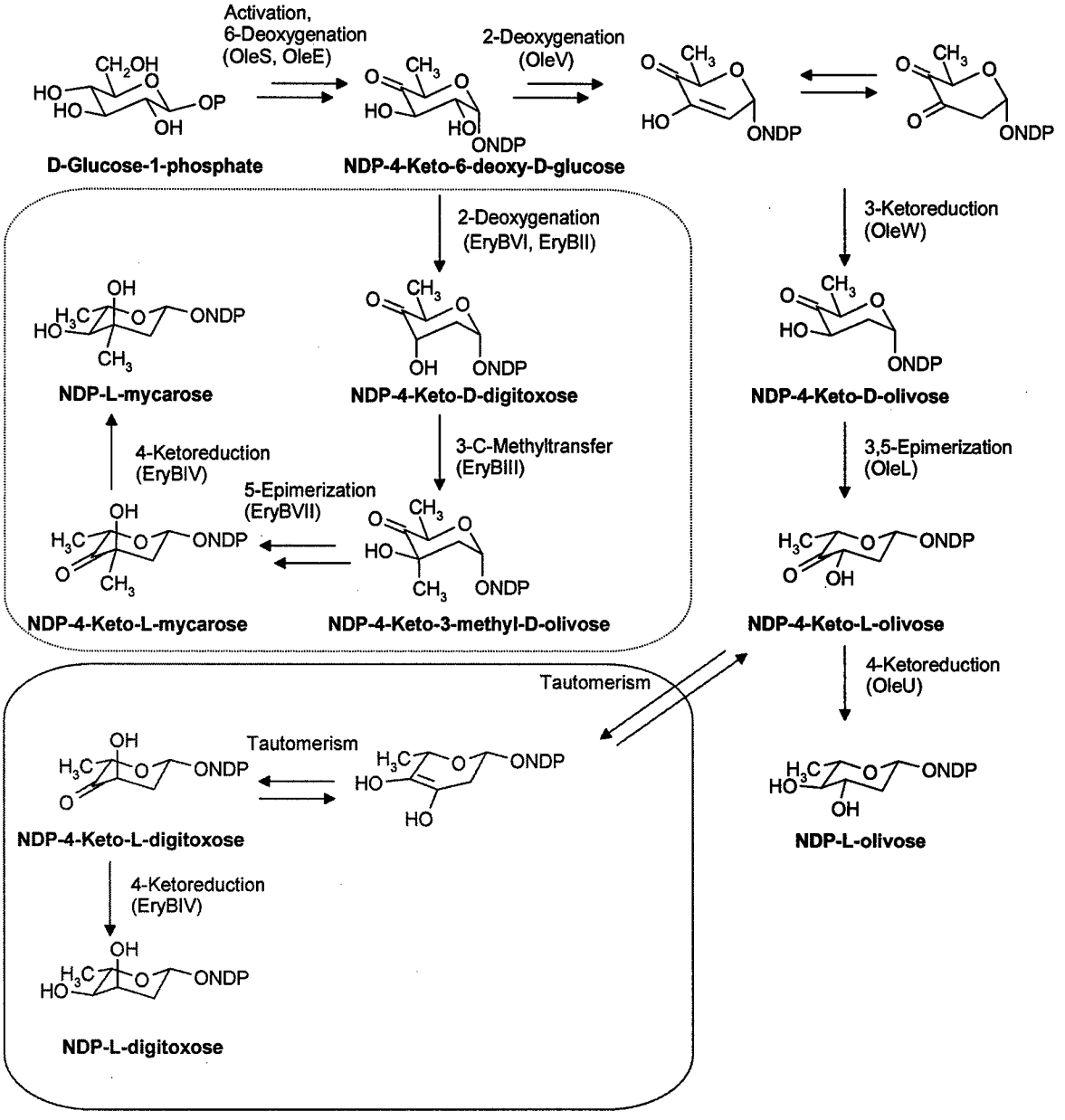

Activation,
6-Deoxygenation
(OleS, OleE)
2-Deoxygenation
(OleV)
D-Glucose-1-phosphate
NDP-4-Keto-6-deoxy-D-glucose
2-Deoxygenation
(EryBVI, EryBII)
3-Ketoreduction
(OleW)
NDP-L-mycarose
NDP-4-Keto-D-digitoxose
NDP-4-Keto-D-olivose
4-Ketoreduction
(EryBIV)
3-C-Methyltransfer
(EryBIII)
3,5-Epimerization
(OleL)
5-Epimerization
(EryBVII)
NDP-4-Keto-L-mycarose
NDP-4-Keto-3-methyl-D-olivose
NDP-4-Keto-L-olivose
Tautomerism
4-Ketoreduction
(OleU)
Tautomerism
NDP-4-Keto-L-digitoxose
NDP-L-olivose
4-Ketoreduction
(EryBIV)
NDP-L-digitoxose

Table 1: ^{1}H and ^{13}C NMR Data of 8-Demethyl-8-L-digitoxosyltetracenomycin C (**4**, d_6-acetone), and 8-Demethyl-8-D-glucosyltetracenomycin C, (**7**, CD_3OD), at 400 MHz and 100.7 MHz, respectively. δ in ppm

Position	**4**	**4**	**7**	**7**
	δ_H multiplicity (*J* in Hz)	δ_C	δ_H multiplicity (*J* in Hz)	δ_C
1		190.8 s		n.o.
2	5.61 s	100.1 d	5.61 s	100.8 d
3		175.4 s		176.1 s
3-OCH_3	3.83 s	57.4 q	3.82 s	57.7 q
4	5.05 d (6)	70.7 d	4.89-4.90[a] m	70.9 d
4-OH	4.93[b] d (6)			
4a		n.o.		n.o.
4a-OH	5.13[b] br. S			
5		194.2 s		193.2 s
5a		141.2 s		141.8 s
6	7.98 s	121.3 d	8.03 s	122.2 d
6a		130.8 s		129.7 s
7	7.74 s	111.7 d	7.67 s	113.1 d
8		155.7 s		156.7 s
9		129.4 s		130.8 s
9-C=O		168.4 s		169.7 s
9-OCH_3	3.95 s	53.2 q	3.97 s	53.4 q
10		138.6 s		138.4 s
10a		121.9 s		123.1 s
10-CH_3	2.83 s	21.2 q	2.84 s	21.3 q
11		167.9 s		n.o.
11-OH	14.03[b] s			
11a		110.3 s		110.6 s
12		198.1 s		198.3 s
12a		83.7 s		84.7 s
12a-OH	5.76[b] br. S			
1'	5.97 d (3.5)	96.3 d	5.20 d (7)	101.9 d
$2'_{ax}$	2.20 ddd (15, 3.5, 3.5)	36.2 t	3.43-3.55[a] m	74.9 d
$2'_{eq}$	2.28 ddd(15, 3, 1)	36.2 t		
3'	4.04 ddd(3.5, 3, 3)[c]	67.1 d	3.43-3.55[a] m	78.3 d
3'-OH	3.34[b] d (6.5)			
4'	3.27 dd (9.5, 3)[c]	73.2 d	3.38 dd (9, 9)	71.4 d
4'-OH	3.71[b] d (7)			
5'	3.93[a] dq (9.5, 6)	66.0 d	3.59-3.65 m	78.6 d
6'	1.17 d (6)	18.3 q		
$6_A'$-CH_2			3.67-3.74 m	62.7 t
$6_B'$-CH_2			3.94-3.97[a] m	62.7 t

[a] Overlapped by other signal. [b] Exchangeable by D_2O. [c] Observed after addition of D_2O
n.o.= not observed

Chemistry & Biology, Vol. 9, 519–531, April, 2002, ©2002 Elsevier Science Ltd. All rights reserved. PII S1074-5521(02)00126-6

The Biosynthetic Gene Cluster for the Antitumor Rebeccamycin: Characterization and Generation of Indolocarbazole Derivatives

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Summary

Rebeccamycin, a halogenated natural product of the indolocarbazole family, is produced by *Saccharothrix aerocolonigenes* ATCC39243. Several rebeccamycin analogues, which target DNA topoisomerase I or II, have already entered clinical trials as anticancer drugs. Using as a probe an internal fragment of *ngt*, a *Saccharothrix aerocolonigenes* gene encoding an indolocarbazole *N*-glycosyltransferase, we isolated a DNA region that directed the biosynthesis of rebeccamycin when introduced into *Streptomyces albus*. Sequence analysis of 25.6 kb revealed genes for indolocarbazole core formation, halogenation, glycosylation, and sugar methylation, as well as a regulatory gene and two resistance/secretion genes. Heterologous expression of subsets of these genes resulted in production of deschloro-rebeccamycin, 4′-demethyl-deschloro-rebeccamycin, and deschloro-rebeccamycin aglycone. The cloned genes should help to elucidate the molecular basis for indolocarbazole biosynthesis and set the stage for the generation of novel indolocarbazole analogues by genetic engineering.

Introduction

Since its discovery in 1977, the indolocarbazole family of natural products has received great attention from both organic chemists and biomedical researchers. The reason for this interest resides in the novel structures and wide range of biological activities (antibacterial, antifungal, and antitumor) that members of this family display [1]. Indolocarbazole natural products are defined by their characteristic structure containing either indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole (e.g., rebeccamycin), indolo[2,3-*a*]carbazole (e.g., tjipanazoles), or *bis*-indolylmaleimide (e.g., arcyriarubin) moieties. In particular, the indolopyrrolocarbazoles constitute a new class of antitumor drugs, which can be divided into two major groups depending on their mechanisms of action and structural features. One group consists of protein kinase inhibitors, such as staurosporine and K-252a. Usually, they contain a sugar moiety linked to both indole nitrogens of the indolocarbazole core. The second group consists of DNA-damaging agents, exemplified by rebeccamycin and AT2433, that act on DNA topoisomerase I or II. Most members of this group contain a sugar moiety attached by a β-glycosidic linkage to only one of the indole nitrogens of the aglycone. It has been shown that the carbohydrate plays a crucial role in the activity of these compounds [2]. Presently, three protein kinase inhibitors (UCN-01, CGP 41251, CEP-751) and two DNA-damaging agents (NB-506, NSC655649) have already entered clinical trials for their use against several types of tumors [3].

Rebeccamycin (Figure 1) is an antitumor antibiotic produced by the actinomycete *Saccharothrix aerocolonigenes* ATCC39243 [4, 5]. It shows antibacterial activity against several Gram-positive bacteria, including *Staphylococcus aureus* and *Streptococcus faecalis*. Rebeccamycin also inhibits the growth of some tumor cell lines and displays activity against several types of tumors implanted in mice [5]. There is a considerable effort placed on the design and synthesis of new rebeccamycin analogs and derivatives with enhanced antitumor activity [6]. From a different point of view, rebeccamycin has also attracted interest because it is a halogenated natural product [7].

Despite the interest focused on indolocarbazoles, very little is known about the biochemical and genetic aspects of their biosyntheses. As far as we know, no complete set of genes for the biosynthesis of an indolocarbazole natural product has been reported. A patent application has claimed the cloning of some genes needed for the last steps in the biosynthesis of the staurosporine sugar moiety in *Streptomyces longisporoflavus* DSM10189 [8]. More recently, the cloning and sequencing of a gene, *ngt*, encoding an indolocarbazole *N*-glycosyltransferase from *Saccharothrix aerocolonigenes* ATCC39243 has been reported [9].

Here we report the cloning and sequencing of the complete gene cluster encoding rebeccamycin biosynthesis from *Saccharothrix aerocolonigenes* ATCC39243. We also show that the identified set of genes is necessary and sufficient for rebeccamycin production by heterologous expression in *Streptomyces albus*. Finally, we report the production of rebeccamycin derivatives in *S. albus* when transformed with selected subsets of the identified genes.

Results and Discussion

Isolation of the DNA Region Surrounding the *ngt* Gene

Recently, the *Saccharothrix aerocolonigenes* ATCC39243 *ngt* gene, encoding an *N*-glycosyltransferase able to introduce a D-glucose moiety into indolocarbazoles

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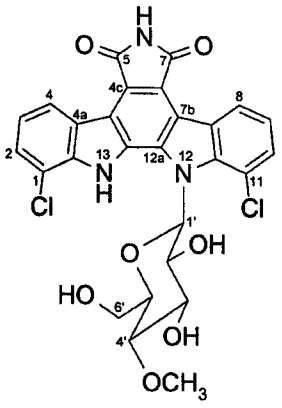
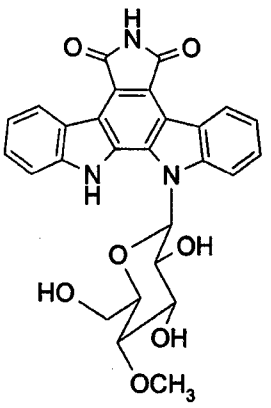
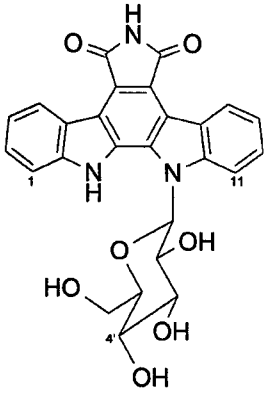


1: Rebeccamycin

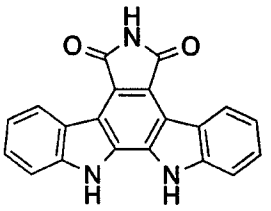


2: Deschloro-rebeccamycin

Figure 1. Structure of Rebeccamycin and Derivatives



3: 4'-Demethyl-deschloro-rebeccamycin



4: Deschloro-rebeccamycin aglycon

J-104303 and 6-*N*-methylarcyriaflavin C, has been cloned and sequenced [9]. No other open reading frame (ORF) was reported in the sequenced region, encompassing 1898 nucleotides (nt). Given the known precedent that antibiotic biosynthetic genes commonly occur as a cluster in antibiotic-producing actinomycetes, we set out to isolate the DNA flanking *ngt* in order to identify the rebeccamycin biosynthetic gene cluster. A *Saccharothrix aerocolonigenes* ATCC39243 genomic library was constructed in the *Escherichia coli-Streptomyces* shuttle vector pKC505 [10]. This library was screened with a probe from an internal fragment of *ngt*, resulting in the isolation of several overlapping cosmids (10A4, 14E8, 17A12, and 24B2). It has been previously shown by PCR amplification that the genome of *Saccharothrix aerocolonigenes* ATCC39243 contains at least one $FADH_2$-dependent halogenase gene [11]. In order to investigate if this putative halogenase gene was part of the rebeccamycin biosynthetic gene cluster, we analyzed the isolated cosmids by PCR with degenerate oligoprimers designed to amplify halogenase genes. A PCR product of the expected size was amplified only from cosmids 14E8 and 17A12. The sequencing of these PCR products confirmed that they were identical to the sequence previously reported [11].

Production of Rebeccamycin in a Heterologous Host

In order to determine if any of the isolated cosmids harboured the complete rebeccamycin gene cluster, we independently introduced the cosmids into the heterologous host *Streptomyces albus* J1074 and analyzed transformants for production of rebeccamycin or any biosynthetic precursor. We routinely use *S. albus* J1074 as a host for heterologous gene expression and metabolite production because this strain shows a nice, dispersed growth, a very low background concerning aromatic metabolite production (as detected by HPLC), and a good efficiency of protoplast transformation. Two of the cosmids (14E8 and 17A12) conferred the ability to produce a compound not produced by the host strain *S. albus* J1074. The identity of this compound with rebeccamycin was demonstrated as follows. First, it showed antibacterial activity in growth inhibition tests performed on *Micrococcus luteus*. Second, it had the same relative mobility as authentic rebeccamycin in

Table 1. ^{1}H NMR data of rebeccamycin (1), deschlororebeccamycin (2), 4′-demethyl-deschlororebeccamycin (3), and deschloro-rebeccamycin aglycon (4) in d_6-DMSO at 400 MHz

	Compound			
	1	2	3	4
Position	δ, Multiplicity (J/HZ)	δ, Multiplicity (J/HZ)	δ, Multiplicity (J/HZ)	δ, Multiplicity (J/HZ)
1-H	–	9.05 d (8)	9.04 d (8)	8.99 d (8)
2-H	7.66 d (8)	7.33 t (8)	7.33 t (8)	7.34 t (8)
3-H	7.44 t (8)	7.54 t (8)	7.50-7.57 t (8)[a]	7.54 t (8)
4-H	9.06 d (8)	7.68 d (8)	7.65 d (8)	7.80 d (8)
N6-H	11.34 s	11.37 s	11.06 s	10.96 s
8-H	9.24 d (8)	7.92 d (8)	7.93 d (9)	7.80 d (8)
9-H	7.44 t (8)	7.54 t (8)	7.50-7.57 dd (9,8)[a]	7.54 t (8)
10-H	7.72 d (8)	7.33 t (8)	7.33 t (8)	7.34 t (8)
11-H	–	9.13 d (8)	9.12 d (8)	8.99 d (8)
N12-H	–	–	–	11.77 s
N13-H	10.66 s	11.57 s	11.63 s	11.77 s
1′-H	6.91 d (9)	6.26 d (9)	6.24 d (9)	–
2′-H	3.69 ddd (9,9,6)	3.52 ddd (9,9,5)	3.50 ddd (9,9,5)[b]	–
2′-OH	5.0 d (6)	4.93 d (5)	4.87 d (5)	–
3′-H	3.56 ddd (9.5,9,6)	3.69 ddd (9.5,9,6)	3.54 ddd (9,9,5)[b]	–
3′-OH	5.39 d (6)	5.24 d (6)	5.09 d (5)	–
4′-H	3.64 dd (9.5,9.5)	3.74 dd (9.5,9.5)	3.96 ddd (9.5,9.5,5)	–
4′-OH	–	–	5.34 d (5)	–
4′-OCH_3	3.59 s	3.62 s	–	–
5′-H	3.82 dt (9.5,5)	3.85 ddd (9.5,5,4)	3.78 ddd (9,4,2)	–
6′-H_2	3.94 dd (6,5)	3.93 dd (11,4) 3.98 dd (11,5)	3.93 dd (11,2)[c] 4.04 dd (11,4)	–
6′-OH	5.27 t (6)	6.10 dd (5,4)	5.96 t (4)	–

δ is given in ppm relative to internal TMS.
[a] Complex, overlapping signals; *J* not exactly determinable.
[b] Overlapping signal assignments are interchangeable.
[c] Broad signal.

HPLC analysis under different chromatographic conditions. Third, it showed the characteristic rebeccamycin absorption spectrum with maxima at 238 and 316 nm. Fourth, the compound was purified and studied by high-resolution EI-MS, yielding a main peak with a mass of 569.0753 corresponding to rebeccamycin with theoretical calculated molecular mass of 569.0757 (for $C_{27}H_{21}N_3O_7Cl_2$), and a minor peak with a mass of 393 corresponding to rebeccamycin aglycone. Similar MS results were previously reported for purified rebeccamycin [4]. Finally, the structure of the product was confirmed to be that of rebeccamycin by NMR analysis as described below.

Rebeccamycin (1, Figure 1) was analyzed by 1D ^{1}H and 2D ^{1}H, ^{13}C NMR spectroscopy. The ^{13}C, ^{1}H HSQC (heteronuclear single quantum coherence) experiment and a 2D ^{13}C, ^{1}H HMBC experiment allowed the detection and assignment of almost all carbons of the molecule (Table 1). For instance, a distinctive proton signal with δ 11.36 couples to the carbons with δ 170.1 (C5 and C7), 123.3 (C7a), and 121.3 (C4c) and apparently belonged to the N bound 6-H of rebeccamycin. The cross peaks at 10.69/137.8, 10.69/130.4, and 10.69/118.3 tied the proton at N-13 to the corresponding carbons 13a, 12b, and 4b of the pyrrole ring. An aromatic proton, part of an ABC system, with δ 9.09 (4-H) was strongly coupled to the carbons with δ values of 137.8 (C13a) and 127.8 (C4a), and the ^{1}H signal with δ 9.27 (8-H) gave intense cross peaks with carbons possessing δ 138.2 (C11a) and 130.7 (C7c).

The signals, typical for a sugar moiety (Tables 1 and 2, C1′ to C6′′, 1′-H to 6′-H_2) and in line with the observations that the nitrogen atom at the 12 position lacks a proton and that carbons 11a and 12a, adjacent to N-12, are coupled to a proton of C1′ of this glycoside residue, confirm the presence of the sugar moiety and its *N*-glycosidic linkage to N-12. The NMR data are close to those reported earlier [4]. Thus, the combination of HSQC, HMBC, and DQCOSY NMR spectra along with the MS data clearly showed the compound at hand to be rebeccamycin (1; Figure 1).

Rebeccamycin production levels in these *S. albus* recombinant strains were several fold greater than those obtained with *Saccharothrix aerocolonigenes* ATCC39243 grown in the same conditions. Cosmid 14E8, one of the cosmids able to direct rebeccamycin biosynthesis, was chosen for further study.

It is known that rebeccamycin has antibacterial activity against some Gram-positive bacteria and that it produces a weak inhibition of growth against some *Streptomyces* spp. [12]. Based on the frequent linkage between biosynthetic and resistance genes, we decided to determine whether the cloned DNA contained rebeccamycin resistance determinants. We checked the effect of exogenously added rebeccamycin on the growth of *S. albus* J1074/14E8 and on that of the control strain *S. albus* J1074/pKC505. Paper disc diffusion assays showed that growth of *S. albus* J1074/pKC505 was totally inhibited by 100 μg rebeccamycin, whereas *S. albus* J1074/14E8 was not affected by amounts of as much as 200 μg.

Table 2. ^{13}C NMR data of rebeccamycin (1)[a], deschloro-rebeccamycin (2), 4′-demethyl-deschloro-rebeccamycin (3), and deschloro-rebeccamycin aglycon (4) in DMSO-d_6 at 100.6 MHz

	Compound			
	1[a]	2	3	4
Position	δ	δ	δ	δ
1	117.0	124.4	125.1	124.1
2	130.4	120.4[b]	121.0[b]	120.1
3	123.1	126.9[b]	127.6[b]	126.6
4	124.1	112.2	112.8[b]	111.9
4a	127.8	121.4[b]	122.1[b]	121.4
4b	118.3	118.6[b]	119.1[b]	115.4
4c	121.3	121.1[b]	121.3[b]	119.7
5	170.1	171.0	171.7	171.1
7	170.1	171.0	171.7	171.1
7a	123.3	119.4[b]	120.1[b]	119.7
7b	119.2*	116.9[b]	117.6[b]	115.4
7c	130.7	121.1[b]	121.7[b]	121.4
8	124.7	111.7	112.5[b]	111.9
9	123.1	126.8[b]	127.4[b]	126.6
10	128.1	120.6[b]	121.7[b]	120.1
11	117.0	124.4	125.1	124.1
11a	138.2	142.1	142.8	140.1
12a	129.5[c]	128.2	129.0	128.9
12b	130.4	129.6	130.4	128.9
13a	137.8	140.7	141.4	140.1
1′	85.2	84.1	85.1	–
2′	72.9	73.1	73.2	–
3′	78.0	76.3	77.3	–
4′	79.7	77.3	68.2	–
4′-OCH_3	60.7	60.0	–	–
5′	80.8	77.1	79.2	–
6′	60.4	58.5	58.9	–

δ is in ppm relative to internal TMS. Assignments were made with the help of couplings observed in the HSQC and HMBC spectra.
[a] Data from HSQC and HMBC spectra.
[b] Assignments are interchangeable within each group.
[c] Not observed by the indirect detection methods, taken from the literature ([4], 90 MHz) for comparison reasons.

Analysis of the Rebeccamycin Biosynthetic Gene Cluster

The complete DNA sequence of the insert contained in cosmid 14E8 was determined to consist of 25,681 nt (the sequence was deposited at the EMBL Nucleotide Sequence Database with accession number AJ414559). Sequence analysis revealed the presence of 16 complete ORFs and two incomplete ORFs, whose organization is shown in Figure 2A. Database comparisons allowed us to propose functions for most of these ORFs (Table 3). Genes apparently involved in rebeccamycin biosynthesis seem to be organized in four transcriptional units: *rebG*, *rebODCPM*, *rebRFUH*, and *rebT*. In operons *rebODCPM* and *rebRFUH*, genes are translationally coupled, with *rebM*, whose start codon is only 26 nt away from *rebP* stop codon, as the only exception. Each of these polycistronic transcriptional units ends with an inverted repeat (nt 17,412–17,447, and nt 23,872–23,927), which could lead to stem-loop secondary structures at the mRNA and transcription termination.

Genes Probably Involved in Formation of the Indolocarbazole Core

Based on sequence analysis and database comparison, we propose a group of four genes, *rebO*, *rebD*, *rebC*, and *rebP*, to be involved in indolocarbazole core formation. Some experimental evidence for that assumption is also presented in this paper (see below). Previous reports have shown that the indolocarbazole core of both rebeccamycin and staurosporine is biosynthesized from two units of L-tryptophan, with the carbon skeleton incorporated intact [13, 14]. Moreover, indolepyruvic acid has been identified as an intermediate of rebeccamycin biosynthesis [15]. In agreement with this, the *rebO* gene product belongs to a family of flavin-containing amine oxidases, including L-amino acid oxidase AIP from the fish *Scomber japonicus* [16] and putative amine oxidase CC1091 from *Caulobacter crescentus* [17]. The protein encoded by *rebO* could catalyze the first step in indolocarbazole biosynthesis, the oxidative deamination of L-tryptophan to yield indolepyruvic acid. Very recently, it has been shown that feeding either 5-fluorotryptophan or 6-fluorotryptophan to cultures of *Saccharothrix aerocolonigenes* induces the production of novel fluorinated analogs of rebeccamycin [18]. This suggests that RebO may accept different tryptophan derivatives for oxidative deamination.

The exact nature of intermediates and reactions between indolepyruvic acid (or 7-chloro-indolepyruvic acid) and rebeccamycin aglycone is unknown, but a pathway including condensation of two tryptophan-derived units, several oxidations, and two decarboxylations seems plausible. Next we describe and discuss genes *rebD*, *rebC*, and *rebP*, probably responsible for these biosynthetic steps.

rebD encodes a protein that showed significant similarities to two database proteins, VioB from *Chromobacterium violaceum* [19, 20] and hypothetical protein SC9A4.17 from *Streptomyces coelicolor* (accession number CAC01644) [21]. VioB is involved in early steps of the biosynthesis of violacein, a blue pigment whose carbon skeleton originates from two molecules of L-tryptophan accompanying decarboxylation [19, 20]. It has been speculated that VioB is a multifunctional enzyme that catalyzes a 1,2-indole shift as well as a condensation reaction between the two tryptophan (or tryptophan-derived) units to generate the violacein pyrrole ring [19]. We propose that RebD performs a similar function in rebeccamycin biosynthesis by condensing two tryptophan-derived units (probably indolepyruvic acid or its 7-chlorinated form) to yield the first *bis*-indole intermediate. However, important functional differences between violacein and indolocarbazole biosynthetic enzymes must exist. The non-indolic nitrogen in violacein exclusively originates from one of the tryptophan units that is incorporated intact; the other tryptophan unit somehow loses its α-amino group and suffers an intramolecular 1,2-indole shift [20]. On the other hand, the origin of the non-indolic nitrogen in indolocarbazoles remains mysterious; it appears that the side-chain nitrogen in tryptophan is cleaved and not incorporated into staurosporine [22].

As mentioned above, some genes needed for biosynthesis of the staurosporine sugar moiety in *S. longisporoflavus* DSM10189 have been previously identified [8]. These authors reported 10 kb of contiguous DNA sequence, deposited as three fragments with accession numbers A60304, A60301, and A60305. However, a de-

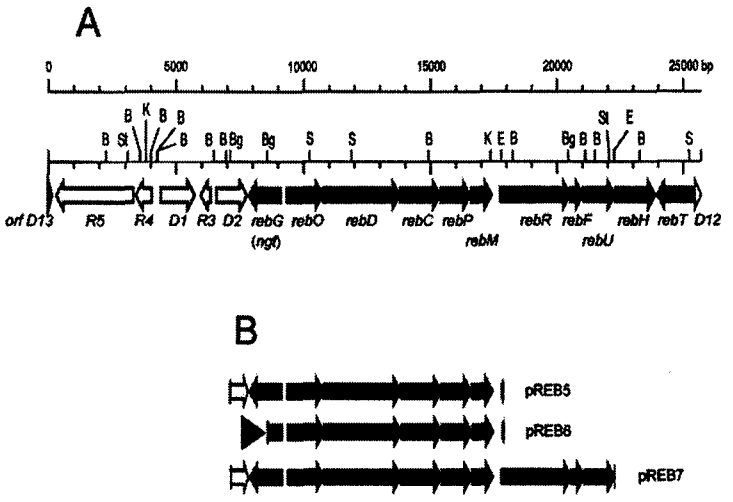


Figure 2. The Rebeccamycin Gene Cluster and Its Heterologous Expression

(A) Restriction map and genetic organization of the *Saccharothrix aerocolonigenes* ATCC39243 DNA region that includes the rebeccamycin biosynthetic gene cluster (in red). Proposed functions for individual ORFs are summarized in Table 1. Sites for restriction enzymes are abbreviated as follows: B, BamHI; Bg, BglII; E, EcoRI; K, KpnI; S, ScaI; and St, StuI.

(B) Schematic representation of inserts contained in plasmids pREB5, pREB6, and pREB7. A green arrow indicates the *ermE**p promoter included in pREB6.

tailed analysis of the complete DNA sequence was not reported, and they identified only the following ORFs in their patent application (but not in their database entry): (1) in accession number A60304—gene 2 (putative sugar *N*-methyltransferase), gene 5 (putative sugar aminotransferase), and three ORFs with no proposed function (genes 1, 3, and 4); (2) in accession number A60301—gene 1 (sugar *O*-methyltransferase) and gene 2 (putative sugar 3,5-epimerase); and (3) in accession number A60305—no ORFs reported. Because we expected that rebeccamycin and staurosporine gene clusters would share some equivalent genes for indolocarbazole core biosynthesis, we decided to perform a detailed sequence analysis of the staurosporine DNA sequence. This analysis revealed a 5′-truncated ORF, previously not reported, at an end of the staurosporine sequence

Table 3. Deduced Functions for Genes in the Rebeccamycin Gene Cluster

Gene	Amino Acids	Closest Similar Protein (% Identity/Similarity), Accession Number	Proposed Function
orfD13	44[a]	–	unknown
orfR5	1003	regulatory protein AfsR from *Streptomyces coelicolor* (36/49), P25941	regulatory protein
orfR4	210	putative dipeptidase SCC53.19 from *S. coelicolor* (52/63), CAB93448	D-alanyl-D-alanine dipeptidase
orfD1	472	hypothetical protein SC6E10.10 from *S. coelicolor* (31/48), CAB51964	secreted esterase
orfR3	133	–	unknown
orfD2	395	hypothetical protein CC0823 from *Caulobacter crescentus* (53/66), AAK22808	unknown
rebG/ngt	421	probable glycosyltransferase from *Deinococcus radiodurans* (45/57), F75587	*N*-glycosyltransferase
rebO	473	L-amino acid oxidase AIP from *Scomber japonicus* (29/44), CAC00499	L-amino acid oxidase
rebD	1013	VioB from *Chromobacterium violaceum* (34/47), AAD51809	*bis*-indole formation
rebC	529	2.4-dihydroxybenzoate monooxygenase from *Sphingomonas* sp. (32/46), CAA51370	FAD-containing monooxygenase
rebP	397	cytochrome P450 YjiB from *Bacillus subtilis* (37/53), O34374	P450 heme-thiolate protein
rebM	273	methyltransferase from *Amycolatopsis mediterranei* (50/66), AAC01738	methyltransferase
rebR	923	transcriptional activator NysRI from *Streptomyces noursei* (25/35), BAB50206	regulatory protein
rebF	170	putative FMN:NADH oxidoreductase Gra-orf34 from *S. violaceoruber* (39/52), CAA09661	flavin reductase
rebU	426	putative integral membrane ion antiporter from *A. orientalis* (40/54), CAB45049	integral membrane transporter
rebH	530	tryptophan halogenase PrnA from *Pseudomonas chlororaphis* (55/72), AAD46360	$FADH_2$-dependent halogenase
rebT	473	putative antibiotic antiporter FrnF from *S. roseofulvus* (44/62), AAC18101	integral membrane transporter
orfD12	81[a]	hypothetical transcriptional regulator from *M. tuberculosis* (43/63), Q10810	regulatory protein

[a]Incomplete ORF.

(nt 1–332 of accession number A60304). The deduced product of this ORF showed similarity to the C termini of *C. violaceum* VioB (48% identity, 57% similarity) and of RebD (63% identity, 70% similarity). The presence of a *vioB/rebD* homolog in the violacein, staurosporine, and rebeccamycin gene clusters reinforces the idea of its involvement in the condensation of two tryptophan-derived units.

Two of the identified genes, *rebC* and *rebP*, could be involved in oxidative conversion of the first *bis*-indole intermediate into the indolocarbazole core. Analysis of *rebC* gene product showed an N-terminal FAD binding domain [23] and clear similarities to a family of FAD-containing, NAD(P)H-dependent monooxygenases acting on aromatic compounds [24]. The highest similarities were found to 2,4-dihydroxybenzoate monooxygenase DxnD from *Sphingomonas* sp. [25] and to putative polyketide hydroxylase SchC from *Streptomyces halstedii* [26]. DxnD exhibits NADH oxidation activity with 2,4-dihydroxybenzoate and is believed to catalyze decarboxylating monooxygenation to yield hydroxyquinol as a product [25]. In our above-mentioned analysis of the staurosporine DNA sequence, we also found a previously unreported ORF (nt 1845–2122 of accession number A60301, plus nt 1–1373 of accession number A60305) whose deduced product showed similarities to the same family of monooxygenases, including *Sphingomonas* sp. DxnD (32% identity, 47% similarity) and RebC (63% identity, 71% similarity). According to this, a monooxygenase such as RebC could catalyze decarboxylative monooxygenations, as shown for *Sphingomonas* sp. DxnD [25]. On the other hand, *rebP* gene product is homologous to P450 heme-thiolate proteins [27]. The highest similarities were found to a putative cytochrome P450 YjiB from *Bacillus subtilis* (accession number O34374) and a probable cytochrome P450 hydroxylase from *S. coelicolor* (accession number T36526). In our analysis of the staurosporine DNA sequence, we found that the deduced product of accession number A60304 "gene 1" (nt 378–1655), of unreported function, was also similar to P450 heme-thiolate proteins, including *B. subtilis* YjiB (32% identity, 51% similarity) and RebP (51% identity, 60% similarity). It is worth mentioning that some P450 enzymes are able to form new C-C or C-O bonds between aromatic moieties by phenol oxidative coupling [28, 29]. One may hypothesize that, in an analogous way, RebP could catalyze oxidative ring closure of a *bis*-indole intermediate to form the indolocarbazole core.

Genes Probably Involved in Indolocarbazole Modification

After (or during) formation of the indolocarbazole core, additional reactions must occur to arrive at the final rebeccamycin molecule. These would include glycosylation and halogenation. A gene encoding an indolocarbazole *N*-glycosyltransferase, *ngt*, has been previously identified from *Saccharothrix aerocolonigenes* [9]. A comparison of the 1898 nt DNA sequence reported for *ngt* (accession number AB023953) [9] and the corresponding sequence determined by us (complementary to nt 7484–9390 of accession number AJ414559) revealed several discrepancies in *ngt/rebG* coding sequence and also in flanking sequences. In particular, the deduced products of *ngt* and *rebG* differ in eight amino acid residues, and RebG includes six additional residues at its C terminus. Some of the discrepancies in the *ngt/rebG* flanking sequences also affect *orfD2* and *rebO*, genes not previously defined, and make them unfunctional in the published *ngt* sequence [9]. After rechecking and confirming our sequence, we find it plausible that these discrepancies originate from sequencing errors in the published *ngt* sequence. So, from now on we use the name *rebG* for the corrected sequence of the previously known *ngt* gene. RebG belongs to "family 1" of NDP-sugar glycosyltransferases [30]. The highest similarities were found to a probable glycosyltransferase from *Deinococcus radiodurans* (accession number F75587) and zeaxanthin glucosyl transferase CrtX from *Synechocystis* sp. (accession number S74500). It has been previously shown that *rebG* (*ngt*) encodes an *N*-glycosyltransferase that, when expressed in *S. lividans*, is able to introduce a D-glucose moiety into indolocarbazoles J-104303 and 6-*N*-methylarcyriaflavin C [9]. We propose that the physiological function of RebG in *Saccharothrix aerocolonigenes* is the catalysis of an *N*-glycosidic bond between a nucleotide-activated D-glucose and the rebeccamycin indolocarbazole core (Figure 3).

A methylation step is required to yield the 4-*O*-methyl-D-glucose moiety present in rebeccamycin. The deduced product of *rebM* is similar to S-adenosylmethionine-dependent methyltransferases, including a methyltransferase from *Amycolatopsis mediterranei* (accession number AAC01738) and MitM from *Streptomyces lavendulae* [31]. RebM is also similar to sugar methyltransferases deduced from the reported staurosporine DNA sequence: *O*-methyltransferase encoded by gene 1 of accession number A60301, nt 845–1684 (36% identity, 53% similarity), and putative *N*-methyltransferase encoded by gene 2 of accession number A60304, nt 1747–2553 (43% identity, 58% similarity). RebM could catalyze a methylation at the 4-hydroxy position of a D-glucose moiety, probably after the glycosylation step during rebeccamycin biosynthesis (Figure 3).

Chlorination in the rebeccamycin pathway is probably carried out by the *rebH* gene product. RebH resembles $FADH_2$-dependent halogenases [7], including tryptophan halogenase PrnA from *Pseudomonas* spp. and *Myxococcus fulvus* [32] and putative tryptophan halogenases from *Caulobacter crescentus* [17]. PrnA requires $FADH_2$, O_2, and chloride ions to regioselectively convert tryptophan into 7-chlorotryptophan. $FADH_2$ is provided by a flavin reductase that reduces FAD with the help of NADH and that can be substituted by the corresponding enzyme from other organisms [7]. Rebeccamycin is chlorinated precisely at indole positions equivalent to that of 7-chlorotryptophan. This halogenation reaction could be catalyzed by RebH, either on tryptophan or on a later intermediate during rebeccamycin biosynthesis (Figure 3, see below). Chloride ions may be substituted by bromide ions for RebH halogenation because bromoindolocarbazole compounds can be isolated from cultures of *Saccharothrix aerocolonigenes* supplemented with potassium bromide [33].

The deduced product of *rebF* belongs to a family of

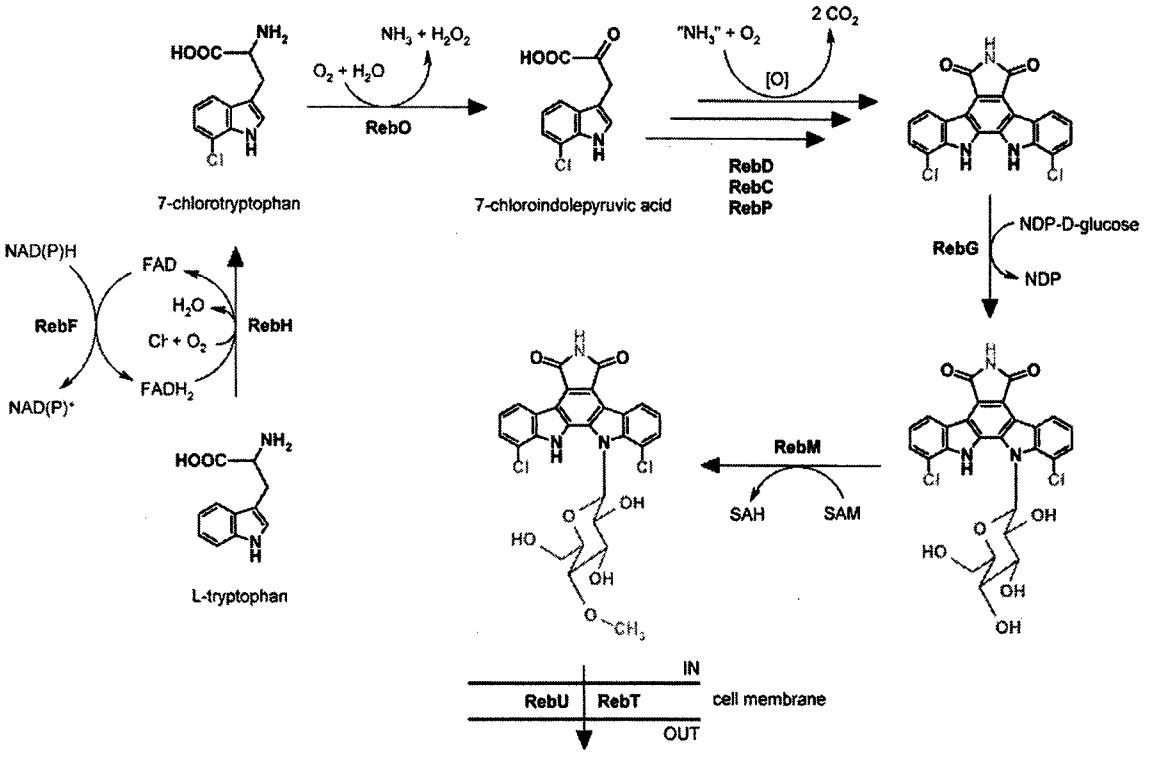


Figure 3. Proposed Biosynthetic Pathway for Rebeccamycin
The relative order of some reactions, such as the halogenation step, may well be different from the one presented here.

flavin:NAD(P)H reductases, the majority of which are part of two-component flavin-diffusible monooxygenase systems [34]. Highest resemblances were found to ActVB homologs from *S. violaceoruber* (Gra-orf34) [35], *S. roseofulvus* (FrnH) [36] and *S. coelicolor* (ActVB) [37], and to StyB protein from *Pseudomonas fluorescens* [38]. ActVB (= ActI-ORF6) is a flavin:NADH oxidoreductase that participates in the last step of actinorhodin biosynthesis, a symmetrical dimerization of two benzoquinone units by a phenolic oxidative coupling. ActVB probably supplies reduced FMN to another as-yet-unknown enzyme directly involved in oxidative chemistry [37]. On the other hand, StyB is a part of stirene monooxygenase, a two-component system (StyAB) responsible for the transformation of stirene to epoxystirene [38]. We propose that RebF and RebH form a two-component halogenase system, in which RebF supplies the reduced, diffusible flavin that RebH needs to function (Figure 3). Very recently, a pair of *rebF-rebH* homologs has been found to be linked in the genome of *S. albogriseolus*, producer of the halogenated metabolite thienodolin (Corina Schmid, personal communication). However, an additional role for RebF in supplying reduced flavin to other rebeccamycin enzymes (RebO, RebC) cannot be ruled out.

Genes Probably Involved in Resistance and Regulation

Two genes, *rebU* and *rebT*, could participate in rebeccamycin resistance and/or secretion. The deduced product of *rebU* is similar to Na^+/H^+ exchange membrane proteins, which contain 10–12 transmembrane regions at the N terminus and a large cytoplasmic region at the C terminus [39]. Characterized members of this family function as antiporters of Na^+ (or K^+) and H^+ and play a key role in maintaining cellular pH and other processes. RebU was found to be most similar to a putative integral membrane ion antiporter from *Amycolatopsis orientalis* (accession number CAB45049) and putative antibiotic transporter AviJ from *S. viridochromogenes* [40]. Very recently, a similar protein (ComF) has been found encoded in the complestatin biosynthetic gene cluster of *S. lavendulae* [41].

The *rebT* gene product, the second candidate for rebeccamycin resistance and/or secretion, belongs to the major facilitator family of integral membrane transporters, responsible for antibiotic or antiseptic efflux with the aid of transmembrane electrochemical gradients [42]. Highest similarities were detected to putative antibiotic antiporter FrnF from *S. roseofulvus* [36] and to putative efflux protein EncT from *S. maritimus* [43]. Plasmid pCS006, which contained *rebT*, was introduced in *S. albus* and was found to confer resistance to rebeccamycin.

Concerning regulation, we found that *rebR* gene product belongs to subfamily LAL of regulatory proteins of the LuxR family, involved in ATP-dependent transcriptional activation [44]. LAL proteins contain an N-terminal ATP binding domain and a C-terminal LuxR-type DNA binding domain. RebR shows low end-to-end similarities to LAL proteins, including transcriptional activator

NysRI from *Streptomyces noursei* [45]. We propose that RebR could function as a transcriptional activator of the expression of rebeccamycin biosynthetic genes, and some experimental evidence (see below) points toward this idea.

Genes Probably Not Involved in Rebeccamycin Biosynthesis

Results from database searching were not significant enough for us to propose functions for *orfD13*, *orfR3*, and *orfD2*. On one hand, deduced gene products of *orfD13* (truncated) and *orfR3* did not have any homologs in the databases, so their possible functions are unknown. On the other hand, OrfD2 is similar to conserved hypothetical proteins from several bacteria, including CC0823 from *Caulobacter crescentus* [17] and SCF41.20c from *S. coelicolor* [21]. It also shows weak, limited similarities to putative 2-hydroxyhepta-2,4-diene-1,7-dioate isomerases such as BH2000 from *Bacillus halodurans* [46], but at the moment we do not feel this is enough to propose a function for OrfD2.

Deduced products of another group of ORFs (*orfR5*, *orfR4*, and *orfD1*) showed similarities to proteins of known functions, but these proteins are apparently unrelated to rebeccamycin biosynthesis. OrfR5 resembles AfsR, a global regulatory protein conditionally required for secondary metabolism in *S. coelicolor* [47]. OrfR4 is similar to putative dipeptidase SCC53.19 from *S. coelicolor* [21] and to *Synechocystis* sp. confirmed D-alanyl-D-alanine dipeptidase SynVanX, probably having a role in cell-wall turnover [48]. Finally, the deduced product of *orfD1* is probably a secreted protein with a signal peptide cleaved between amino acids 47 and 48 (as predicted by the SignalP program [49]) and resembles hypothetical protein SC6E10.10 from *S. coelicolor* [21] and fusidic-acid esterase FusH, an extracellular enzyme from *S. lividans* [50].

At the right end of the sequenced region (Figure 2A), *orfD12* (incomplete) encodes the N-terminal part of a protein homologous to MarR family regulatory proteins, many of which respond to phenolic compounds [51]. Highest similarities were found to hypothetical transcriptional regulators Rv2887 from *Mycobacterium tuberculosis* [52] and SC1A4.04 from *S. coelicolor* [21]. The involvement of OrfD12 in gene regulation for rebeccamycin biosynthesis is unclear because rebeccamycin genes are efficiently expressed from cosmid 14E8, in which *orfD12* is truncated.

Production of Rebeccamycin Derivatives in a Heterologous Host

In order to confirm some of the gene functions deduced by sequence analysis and to produce some rebeccamycin biosynthetic intermediates, we constructed several plasmids containing fragments of cosmid 14E8 (Figure 2B). These multicopy plasmids were introduced into *S. albus* J1074 and, after cultivation of these recombinant strains in the presence of thiostrepton (resistance marker in the vector), extracts of the new strains were analyzed by HPLC for production of indolocarbazole derivatives. The first plasmid, pREB5, contained genes *rebG*, *rebO*, *rebD*, *rebC*, *rebP*, and *rebM*, with their own promoter and/or regulatory sequences. On the basis of the gene functions present in pREB5, it was expected that the plasmid would direct the biosynthesis of deschloro-rebeccamycin. However, transformants *S. albus* J1074/pREB5 did not produce any detectable indolocarbazole (Figure 4A). Because this could have been a consequence of some missing regulatory factor causing poor expression of the cloned genes, two new plasmids, pREB6 and pREB7, were constructed (Figure 2B). Plasmid pREB6 contained genes *rebO*, *rebD*, *rebC*, *rebP*,

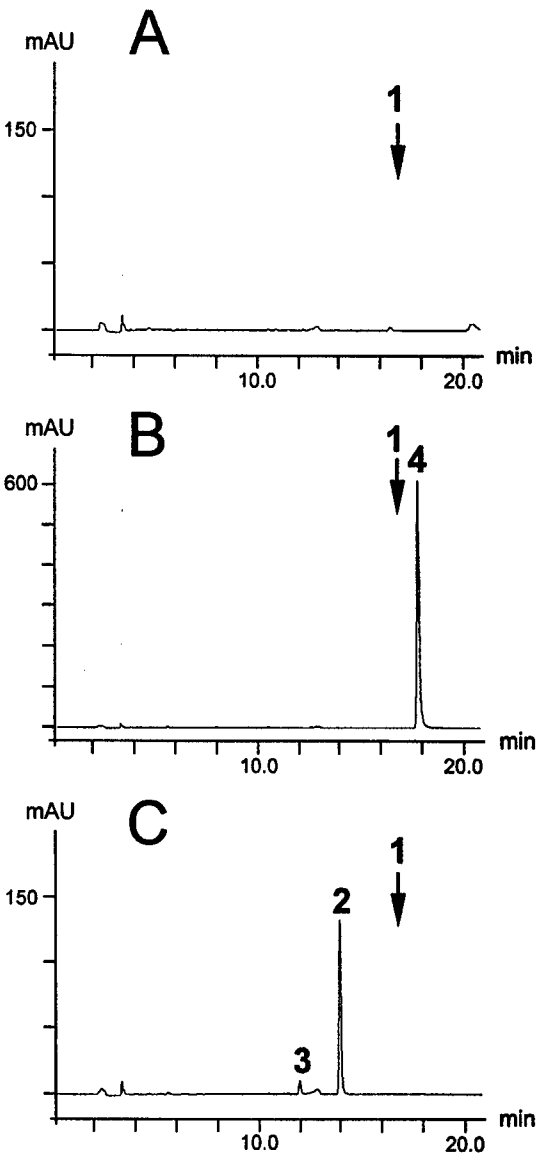


Figure 4. Heterologous Expression of Selected Subsets of Genes from the Rebeccamycin Cluster

HPLC analysis of extracts from (A) *S. albus* J1074/pREB5 (the control *S. albus* J1074/pEM4 showed an identical chromatogram), (B) *S. albus* J1074/pREB6, and (C) *S. albus* J1074/pREB7. The elution time of rebeccamycin, used as a standard and previously purified from *Saccharothrix aerocolonigenes*, is indicated by an arrow. Chemical structures of compounds 1–4 are shown in Figure 1.

and *rebM*, under the control of the strong and constitutive promoter *ermE**p [53], so it was expected to direct the biosynthesis of nonchlorinated rebeccamycin aglycone. Plasmid pREB7 included genes *rebG*, *rebO*, *rebD*, *rebC*, *rebP*, *rebM*, *rebR*, *rebF*, and a 3′-truncated *rebU*, and we expected it could produce deschloro-rebeccamycin. HPLC analysis (Figures 4B and 4C) of extracts from transformants *S. albus* J1074/pREB6 and *S. albus* J1074/pREB7 indeed revealed three new products with characteristic indolocarbazole absorption spectra.

These new biosynthetic products were purified by preparative HPLC and characterized by high-resolution MS and NMR, confirming them as being deschloro-rebeccamycin aglycone (from *S. albus* J1074/pREB6), deschloro-rebeccamycin, and demethyl-deschloro-rebeccamycin (the last two products are from *S. albus* J1074/pREB7), respectively (Figure 1).

The putative aglycone showed a main peak with an m/z value of 325.0845, which corresponds to the calculated molecular mass of the nonchlorinated, deglycosylated rebeccamycin derivative, 325.0851. Interestingly, this ion was also present in the spectra of all the other products analyzed in this study. The ^{13}C NMR experiment showed only ten signals, as expected because of the symmetry of the molecule (Table 1, compound 4), and none of the typical carbohydrate carbons with δ in the range of 60–90 was present in the spectrum of this compound. The HSQC and HMBC experiment allowed the assignments of most carbons; however, the assignments of C1 and C4 and those of C2 and C3 may be interchanged. The integrals of the ^1H NMR spectrum along with the MS data clearly reveal the symmetry of the molecule because each signal corresponds to two protons, except the one of the central N6-H. The presence of the new doublet at δ 8.96 (d, 2H, 1-H, and 11-H), which is part of an ABCD system, proves that the aromatic rings contain here four adjacent protons, which proves that no chlorine atoms are any longer attached to the 1 and 11 positions. Thus, the NMR data also confirm the proposed structure, deschloro-rebaccamycin aglycone (4, Figure 1).

The second compound gave a molecular ion with a high-resolution m/z ratio of 501.1543, which is in agreement with the molecular mass of deschloro-rebeccamycin (calculated for $C_{27}H_{23}N_3O_7$: 501.1536). The broadband-decoupled ^{13}C NMR spectrum of the compound shows all the expected signals; most of the assignments were deduced from the comparison with rebeccamycin and from the 2D homonuclear and heteronuclear correlation experiments (HSQC, HMBC, and DQCOSY, Table 1). As indicated from the NMR data, a 4-O-methyl glucose residue was clearly present in the structure of the metabolite. The data show in particular that the compound contains only two nitrogen bound protons (N13-H and N6-H); the latter shows—as in all compounds described here—a strong coupling with the characteristic carbonyl signal at $\delta_C \approx 171$ in the HMBC spectrum. Also, the usual cross peaks of N13-H with C12b/C13a were observed, as well as the couplings of C1′-H of the 4-O-methyl glucose moiety with C12a and C11a. The protons attached at C1 and C11 ruled out the presence of chlorine atoms at the corresponding positions. These data combined with the MS data prove the structure of deschloro-rebeccamycin (2, Figure 1).

The third isolated compound was a minor rebeccamycin derivative that had a demethylated sugar residue and also lacked both chlorine atoms. This follows from a high-resolution EI-MS experiment, in which the compound gave a molecular ion with m/z ratio of 487.1378, and the prominent aglycone peak at m/z 325 found also in the MS spectra of all the other analyzed products. The high-resolution mass of 487.1378 is in agreement with the calculated mass for $C_{26}H_{20}N_3O_7$ of 487.1380. Unlike rebeccamycin, the compound lacks both chlorine atoms because both the 1-H and the 11-H signals are present in the ^1H NMR spectrum. Also obvious from the ^1H NMR spectrum was that the O-methyl group in the 4′ position is missing. Instead, a new hydroxyl group at δ 5.34 (4′-OH) was found in close proximity to 4′-H (δ 3.96), as indicated by the H,OH coupling observable in the ^1H- and H,H-DQCOSY spectrum. A coupling between 4′-OH and C4′ (δ_C 68.2) was also detected in the HMBC spectrum. All other signals are very similar to those of compound 2. Also, the ^{13}C NMR spectrum (Table 1, compound 3) of this third new compound is very similar to the one of deschloro-rebeccamycin; the only differences are that the signal of C4′ (δ 68.2) is shifted upfield approximately 10 ppm, in agreement with the a missing OCH_3 group in this position, and that there is no signal from an OCH_3 group. These data in combination with the high-resolution MS data prove that this third compound is indeed 4′-demethyl-deschloro-rebeccamycin (3). The occurrence of a minor rebeccamycin derivative that is glycosylated and not methylated may seem illogical because plasmid pREB7 included the putative methyltransferase gene *rebM*. However, similar results have been previously obtained when *Saccharothrix aerocolonigenes* ATCC39243 was fed with unnatural substrates; bioconversion of indolocarbazole aglycone J-104303 yielded the corresponding glycosylated (but not methylated) compound [9], and feeding either 5-fluorotryptophan or 6-fluorotryptophan resulted in production of the corresponding fluorinated 4′-demethyl-deschloro-rebeccamycins or a mixture of both methylated and nonmethylated derivatives [18].

The fact that deschloro-rebeccamycin was produced by a strain harbouring pREB7, but not by a strain carrying pREB5, suggests that transcription of *rebODCPM* probably needs to be activated by the *rebR* product. In the case of pREB6, the strong promoter *ermE**p overcomes this problem because of its constitutive expression. However, *rebG* transcription might not need to be activated by RebR because *rebG* (*ngt*) was expressed in *S. lividans* without any additional promoters [9], although this could also be explained by read-through transcription from vector promoter sequences. The structures of the isolated rebeccamycin derivatives reinforce the functions proposed for the *reb* genes from sequence analysis and database comparisons.

Other laboratories have previously used chemical synthesis or hemisynthesis to obtain compounds structurally identical to nonchlorinated rebeccamycin aglycone and deschloro-rebeccamycin [6]. These authors analyzed the biological activities of these compounds in antimicrobial and antiproliferative tests, as well as in inhibition assays toward protein kinases C (PKC) and A (PKA) and topoisomerases I (Topo I) and II (Topo II) [6,

12]. No inhibition of Topo II was detected with any of the two compounds, as is the case for rebeccamycin itself. Nonchlorinated rebeccamycin aglycone did not show any antimicrobial activity against the different microorganisms tested, including two *Streptomyces* spp. [12]. This result may explain why strain *S. albus* J1074/pREB6 is able to produce this compound and survive, despite the fact that no rebeccamycin resistance or transporter gene is included in pREB6. In the same report, dechlorinated rebeccamycin showed no growth-inhibitory effect against the microorganisms tested, with the only exception being a very weak activity toward one of the two *Streptomyces* spp. assayed [12]. Strain *S. albus* J1074/pREB7 may be protected from the weakly toxic effects of deschloro-rebeccamycin by a 3′-truncated *rebU*, included in pREB7, which encodes an almost complete putative transporter (lacking only 13 amino acids) that could participate in metabolite secretion/resistance. Interestingly, when compared to rebeccamycin, dechlorinated rebeccamycin showed a higher inhibitory activity toward Topo I and similar in vitro antiproliferative activity against murine B16 melanoma and P388 leukemia cells [6]. From the study of a range of glycosylated and nonglycosylated rebeccamycin semi-synthetic derivatives, it has been concluded that the presence of the chlorine atoms on the indole residues is detrimental to both PKC and Topo I inhibitory activities [6]. In this respect, we have shown in this work that the expression of some rebeccamycin biosynthetic genes allows the formation of three nonchlorinated rebeccamycin derivatives that are potentially useful as lead compounds for further drug development. It is also worth mentioning that nonchlorinated rebeccamycin aglycone has comparatively poor antiproliferative and anti-Topo I activities, but it shows some inhibition toward PKC and PKA (whereas neither rebeccamycin nor dechlorinated rebeccamycin inhibit protein kinases). In this sense, it seems that the sugar residue attached to the indolocarbazole chromophore is critical for the drug ability to interfere with Topo I as well as for the formation of intercalation complexes with the DNA [6]. Furthermore, nonchlorinated rebeccamycin aglycone is structurally identical to arcyriaflavin A, a natural product isolated from the slime mold *Arcyria denudata* [54] and the marine ascidian *Eudistoma* sp. [55]. Recently, it has been reported that arcyriaflavin A is a potent inhibitor of human cytomegalovirus replication in cell culture, which could eventually lead to a new class of drugs [56, 57].

With the present work, the utility of manipulating the rebeccamycin biosynthetic genes to produce a variety of indolocarbazole compounds with different biological properties becomes clear. Further experiments are now in progress to produce novel indolocarbazole derivatives and to achieve a better understanding of the biochemical processes involved in rebeccamycin biosynthesis.

Significance

Several indolocarbazoles derived from natural products are presently undergoing phase I/II clinical trials for anticancer chemotherapy, based on their activity as inhibitors of DNA topoisomerases or protein kinases. Additionally, a great interest is focused on the synthesis of a growing number of new indolocarbazole analogs with promising anticancer, antiviral, antifungal, antihypertensive, or neuroprotective activities. Complementary to the efforts in chemical synthesis, the genetic manipulation of genes governing indolocarbazole biosynthesis offers a promising alternative for preparation of these compounds. This work represents the first characterization of a complete gene cluster governing the biosynthesis of an indolocarbazole metabolite, rebeccamycin. Our results are consistent with precursor incorporation studies showing that rebeccamycin is biosynthetically derived from two units of L-tryptophan, one D-glucose, and one L-methionine in *Saccharothrix aerocolonigenes*. Several genes encoding enzymes with unusual or poorly understood activities have been identified, among them those involved in *bis*-indole/indolocarbazole formation, *N*-glycosylation, and halogenation. In addition, genes responsible for indolocarbazole core formation are of special interest because they might be useful as probes for identifying related natural-product biosynthetic genes from other organisms. The availability of the *reb* gene cluster should facilitate future attempts, using combinatorial biosynthesis and rational metabolic pathway engineering, to produce novel indolocarbazoles with improved, or even new, therapeutic activities. The advantage of having this information has already been demonstrated through heterologous expression of (1) the complete gene cluster, which provided a several-fold increase in rebeccamycin production and (2) selected subsets of genes, which allowed us to obtain three different rebeccamycin-related compounds.

Experimental Procedures

Bacterial Strains, Culture Conditions, and Vectors

Saccharothrix aerocolonigenes ATCC39243, *Streptomyces albus* J1074 [58], *Escherichia coli* XL1-Blue [59], *E. coli* ED8767 [60], and *Micrococcus luteus* ATCC1024 were used in this work. Vectors pKC505 [10], pWHM3 [61], and pEM4 [62] have been described previously, and LITMUS 28 (New England BioLabs) was from a commercial source. Shuttle vector pUWL201 was obtained from U. Wehmeier and W. Piepersberg (Wuppertal, Germany). For sporulation, *Saccharothrix aerocolonigenes* and *S. albus* were routinely grown for 7 days at 30°C on agar plates containing either A medium [63] or Bennett's agar [64]. When antibiotic selection of transformants was needed, 100 μg/ml ampicillin, 25 μg/ml thiostrepton (5 μg/ml in liquid media), 25 μg/ml apramycin, or 20 μg/ml tobramycin was used.

DNA Manipulation

Total DNA isolation, plasmid DNA preparations, restriction endonuclease digestions, ligations, and other DNA manipulations were performed according to standard procedures for *E. coli* [65] and for *Streptomyces* [64]. A genomic library of *Saccharothrix aerocolonigenes* was constructed in pKC505 according to literature procedures [10] and screened with a probe made from the *N*-glycosyltransferase gene, *ngt* [9]. This probe was obtained by polymerase chain reaction (PCR) according to standard procedures. Total DNA from *Saccharothrix aerocolonigenes* with oligoprimers CS003 (5′-*TAGAATT*CATCGAACCCGCGGCC-3′, altered sequence in italics, EcoRI underlined) and CS004 (5′-*TATAAGCTT*CGGCTGCCA GCGCTC-3′, altered sequence in italics, HindIII underlined), de-

signed to amplify a DNA fragment encompassing nt 552–1146 from the published *ngt* sequence, was used [9]. Digoxigenin labeling of DNA probes, Southern analysis, hybridization, and detection were performed according to literature procedures [65] and manufacturer recommendations (Boehringer Mannheim). Transformation of *S. albus* protoplasts followed procedures routinely used for *Streptomyces* [64].

PCR amplification of halogenase-encoding regions was performed with degenerate oligoprimers trpst+ (5′-TATCGGATCCGG STGGACCTGGRASATYCC-3′, S = (C, G), R = (A, G), Y = (C, T), BamHI site underlined) and trpst- (5′-AGTTGGTACCGSGCSGCG TASAKGAAGTA-3′, K = (G, T), KpnI site underlined), as previously described [11].

Construction of Plasmids

Cosmid 14E8 was partially digested with BglII and EcoRI, and fragments were subcloned in LITMUS 28, yielding (among others) plasmids pREB1, pCS002, pCS003, and pCS004. One of the resulting plasmids, pREB1, contained a BglII-EcoRI DNA fragment encompassing nt 7,119–17,783 of the sequenced region. This 10.6 kb DNA fragment was obtained from pREB1 as a SpeI-EcoRI fragment and transferred into the XbaI-EcoRI sites of pWHM3, yielding plasmid pREB5. Plasmid pCS002 contained a BglII-EcoRI DNA fragment encompassing nt 8,562–17,783 of the sequenced region. This 9.2 kb DNA fragment was obtained from pCS002 as a BglII-EcoRI fragment and transferred into the BamHI-EcoRI sites of pEM4, yielding plasmid pREB6. Plasmids pCS003 and pCS004 contained, respectively, an EcoRI-BglII DNA fragment (nt 17,783–20,425) and a BglII-EcoRI DNA fragment (nt 20,425–22,241). These two DNA inserts were obtained from pCS003 and pCS004 as EcoRI-BglII and BglII-EcoRI fragments, respectively, and ligated to EcoRI-digested pREB5, resulting in plasmid pREB7.

Plasmid pCS006 was generated by cloning an EcoRI-XbaI DNA fragment from cosmid 14E8 into vector pUWL201. This fragment, which included *rebT*, spanned from nt 22,241 to the end of the insert (nt 25,681) plus 500 bp from pKC505.

DNA Sequencing and Analysis

DNA sequencing was performed on double-stranded DNA templates in pUC18 with the dideoxynucleotide chain termination method [66] and the Cy5 AutoCycle Sequencing Kit (Pharmacia Biotech). An ALF-express automatic DNA sequencer (Pharmacia) was used to sequence both DNA strands with primers supplied in the kit or with internal oligoprimers (17-mer). Computer-aided database searching and sequence analysis were carried out with the University of Wisconsin Genetics Computer Group software [67] and the BLAST program [68].

Tests for Rebeccamycin Antibacterial Activity

Growth inhibition tests on *Micrococcus luteus* were performed to show antibacterial activity of the rebeccamycin present in extracts from *S. albus* transformants. For this, aliquots of acetone extracts were added to paper discs placed on agar plates of TSB (Oxoid) at half nutrient concentration; the plates were preseeded with *M. luteus*. After 2 hr at 4°C, incubation was carried out at 37°C overnight. Rebeccamycin antibacterial activity was also tested against *S. albus* J1074/14E8 and the control *S. albus* J1074/pKC505. For this, different amounts of rebeccamycin (dissolved in acetone) were added to paper discs placed on plates of Bennett's agar (containing 25 μg/ml apramycin) preseeded with the corresponding *S. albus* strain. After 2 hr at 4°C, incubation was carried out at 30°C for 4 days.

HPLC Analysis

Detection of rebeccamycin and related compounds was performed by HPLC in a reversed-phase column (Symmetry C18, 4.6 × 250 mm, Waters), with acetonitrile and 0.1% trifluoroacetic acid in water as solvents. A linear gradient from 20% to 75% acetonitrile in 20 min, at a flow rate of 1 ml/min, was used. Detection and spectral characterization of peaks were performed with a photodiode array detector and Millennium software (Waters), and bidimensional chromatograms were extracted at 316 nm.

Isolation of Rebeccamycin and Related Compounds

Spores of strains *S. albus* J1074/14E8, *S. albus* J1074/pREB6, and *S. albus* J1074/pREB7 were inoculated in TSB medium (Oxoid) and incubated for 24 hr at 30°C and 250 rpm. Each seed culture was used to inoculate (at 2.5%, v/v), eight 2 liter Erlenmeyer flasks containing 400 ml of R5A medium (described as "modified R5 medium" in [63]). After incubation for 5 days in the above conditions, the cultures were centrifuged (12,000 rpm, 30 min). In the cultures of strain *S. albus* J1074/pREB7, rebeccamycin-related compounds were found both in the supernatants and in the pellets, whereas in the other two strains these compounds were largely associated with the pellets; therefore, their supernatants were discarded. The pellets corresponding to each strain were extracted with 400 ml acetone and shaken for 2 hr, after which the suspensions were centrifuged and the organic extract was evaporated in vacuo. The *S. albus* J1074/pREB7 culture supernatant was filtered and applied to a solid-phase extraction cartridge (Sep-Pak Vac 35 cc, Waters). The retained material was eluted with methanol and water, with a linear gradient from 0% to 100% methanol in 1 hr at 10 ml/min. Fractions were taken every 5 min and analyzed by HPLC. The rebeccamycin-related compounds produced by this strain eluted in the fraction taken between 40 and 45 min, which was evaporated in vacuo.

For purification, the material extracted from pellets of each strain was redissolved in 5 ml of a mixture of DMSO and acetone (50:50). The extract from strain *S. albus* J1074/14E8 was chromatographed in a μBondapak C18 radial compression cartridge (PrepPak Cartridge, 25 × 100 mm, Waters) via isocratic elution with acetonitrile and water (55:45) at 10 ml/min. The extract from strain *S. albus* J1074/pREB6 was chromatographed in a semipreparative column (XTerra RP18, 7.8 × 300 mm, Waters) via isocratic elution with methanol and water (90:10) at 3 ml/min. The material extracted from either the pellets or the broth of strain *S. albus* J1074/pREB7 was chromatographed on the semipreparative column mentioned above and eluted with acetonitrile and water (50:50) in isocratic conditions. The compounds collected in every case after multiple injections were dried in vacuo and finally lyophilized.

MS and NMR Methods

NMR spectra were recorded in d6-DMSO on a Varian Inova 400 instrument at 400 MHz for 1H and 100.6 MHz for 13C by the use of 1D spectra and 2D homo- and heteronuclear correlation experiments (1H, 13C, H,H-COSY, HSQC, and HMBC).

Electron ionization mass spectrometry (EI-MS) was used to determine the mass of the purified compounds. The high-resolution electron ionization (HR-EI) mass spectra were acquired at the University of South Carolina, Department of Biochemistry and Chemistry facilities in Columbia, SC with a VG70SQ double focusing magnetic sector MS instrument.

Acknowledgments

The authors wish to thank K.H. van Pée, S. Zehner, and C. Schmid for providing us primers for halogenase genes and for facilitating information prior to publication. We thank the U.S. Department of Defense for support of J.R. et al. and Obra Social Cajastur for financial support to C.S. We acknowledge Drs. W. Cotham and M. Walla (University of South Carolina) for providing the high-resolution MS spectra.

Received: January 11, 2002
Revised: February 12, 2002
Accepted: February 14, 2002

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Accession Numbers

The complete DNA sequence of the insert contained in cosmid 14E8 has been deposited at the EMBL Nucleotide Sequence Database with accession number AJ414559.

Synthesis, Pharmacokinetics, Efficacy, and Rat Retinal Toxicity of a Novel Mitomycin C-Triamcinolone Acetonide Conjugate

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JOURNAL OF
MEDICINAL CHEMISTRY®

Reprinted from
Volume 45, Number 5, Pages 1122–1127

Synthesis, Pharmacokinetics, Efficacy, and Rat Retinal Toxicity of a Novel Mitomycin C-Triamcinolone Acetonide Conjugate

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Received November 5, 2001

A novel conjugate of mitomycin C (MMC) and triamcinolone acetonide (TA) was synthesized using glutaric acid as a linker molecule. To determine the rate of hydrolysis, the conjugate was dissolved in aqueous solution and the rate of appearance of free MMC and TA was determined by high-performance liquid chromatography analysis. Antiproliferative activity of the MMC−TA conjugate and parent compounds was assessed using an NIH 3T3 fibroblast cell line. Cell growth was quantified using the MTT assay. Kinetic analysis of the hydrolysis rate demonstrated that the conjugate had a half-life of 23.6 h in aqueous solutions. The antiproliferative activities of the MMC−TA conjugate and MMC were both concentration dependent, with similar IC_{50} values of 2.4 and 1.7 μM, respectively. However, individual responses at concentrations above 3 μM showed that the conjugate was less active than MMC alone. TA alone showed only limited inhibition of cell growth. Studies evaluating intravitreal injection of the conjugate demonstrate that this agent produced no measurable toxicity. Our data provide evidence that the MMC−TA conjugate could be used as a slow-release drug delivery system. This could in turn be used to modulate a posttreatment wound healing process or to treat various proliferative diseases.

Introduction

Mitomycin C (**1**; MMC) is an antitumor antibiotic with an unusual tetracyclic mitosan ring system, including an aziridine ring, a pyrrolizidine ring, a pyrrolo-(1,2a)-indole, and a substituted benzoquinone moiety.[1−3] Each of these structural elements participates in the unique mechanism-of-action of mitomycin, which is initiated by a reduction of the benzoquinone system leading to a quinone methide covalently binding to DNA and ultimately causing DNA cross-linking and cell death. An initial reduction reaction occurs preferentially in hypoxic cells, which is important for selective antitumor toxicity since many tumors and proliferative disorders are associated with hypoxia.[3b] In addition, mitomycin can alkylate the DNA at the 2-amino group of guanine nucleosides and cause single-strand breakage of DNA as well as chromosomal breaks. MMC (**1**) is used in combination with other antitumor drugs to treat pancreatic carcinoma as well as lymphatic leukemia.[4−7] In addition, MMC has applications in ophthalmology, such as postoperative treatment of the pterygium and adjunctive treatment in glaucoma surgery.[8]

Corticosteroids, such as triamcinolone acetonide (**2**; TA), have only limited antiproliferative properties;[9] however, they may play an important role by preventing the access of inflammatory mediators to a disease site. T-lymphocytes have been, for example, identified in excised membranes from cases of proliferative vitreoretinopathy (PVR), which is scar tissue formation in eyes with rhematogenous retinal detachment.[10,11] Also, elevated levels of certain cytokines, such as tumor necrosis factor-α (TNF-α)[12] and interleukin-1 (IL-1),[13] have been found in the vitreous of patients with proliferative disorders. Glucocorticoids inhibit TNF-α and IL-1 expression[14,15] and can inhibit T-lymphocyte proliferation.[16] Despite the advantages of corticosteroids in the suppression of the inflammatory arm of the wound-healing response, they are limited in their clinical effectiveness because of their weak antiproliferative properties.

The efficacy of antimetabolites and corticosteroids is limited by their short half-life in cavitary organs (e.g., vitreous cavity of the eye). A new slow-delivery system is discussed by Berger and colleagues,[17] utilizing codrug technology. They investigated the use of a 5-fluorouracil and TA conjugate to inhibit experimental PVR in an animal model. In our study, we have investigated a conjugate consisting of MMC and TA. The MMC−TA conjugate (**5**) was synthesized by linking TA and MMC via a glutaric acid unit (Figure 1). In vivo, dissolution of the conjugate followed by hydrolysis allows both active components to be released in an equimolar ratio. We describe the synthesis of the MMC−TA conjugate (**5**), its kinetics, in vitro efficacy, and toxicity to rat retina.

Results

Chemistry. The conjugate of MMC and TA was synthesized in reasonable yields via the route shown in Figure 1. This involved three steps: (i) the catalyzed ketal formation of the secondary (16-OH) and tertiary alcohol (17-OH) groups of triamcinolone with acetone; (ii) the esterification of the primary alcohol (21-OH) of

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10.1021/jm010511b CCC: $22.00
Published on Web 02/05/2002

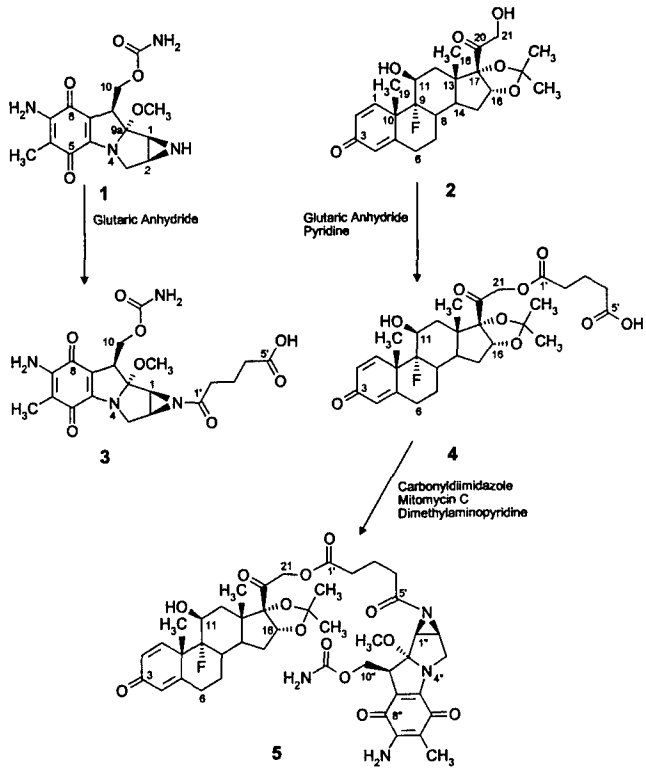

Figure 1. MMC–TA (**5**) was synthesized from the reaction of TA (**2**), the TA glutaric acid-linker molecule (**4**), and MMC (**1**). For the hydrolysis studies, the MMC glutaric acid-linker molecule (**3**) was synthesized from the reaction of MMC (**1**) with glutaric acid.

the resulting TA with the glutaric acid linker using glutaric anhydride and pyridine; and (iii) the amide bond formation between the remaining free, activated (with carbonyl diimidazole) carboxyl group of the glutaric acid linker and the secondary amine group of the aziridine ring of MMC. The resulting MMC–TA conjugate (**5**) was characterized by mass spectrometry and by 1D and 2D nuclear magnetic resonance (NMR) spectroscopy. All obtained data, in particular NMR long-range couplings (see Discussion), verified the conjugate's structure (**5**). For comparative reasons, in context with the hydrolysis studies (see later), MMC–glutaric acid amide (**3**), subsequently called MMC–linker complex, was synthesized from MMC and glutaric anhydride. Structure **3** was verified by its NMR data.

Kinetics and Hydrolysis Studies. In aqueous solutions at pH 7.4, the MMC–TA conjugate was hydrolyzed by cleavage of either the amide bond linking mitomycin and position 5′ of the glutaric acid linker or the ester bond linking TA to the 1′ position of the linker. Therefore, four different compounds were released, (i) MMC (**1**), (ii) TA (**2**), (iii) TA–linker complex (**4**), and (iv) MMC–linker complex (**3**). Peaks for MMC, TA, MMC–linker complex, TA–linker complex, and the MMC–TA conjugate were identified by high-performance liquid chromatography (HPLC) through direct comparison with these molecules, which were available from the synthesis outlined above (Figure 1).

Regression analysis of the disappearance of MMC–TA conjugate in aqueous solution demonstrated that the conjugate has a half-life of approximately 23.6 h (Figure 2). Evolution of MMC and the MMC–linker complex (retention times of 3.07 and 3.26 min, respectively) and

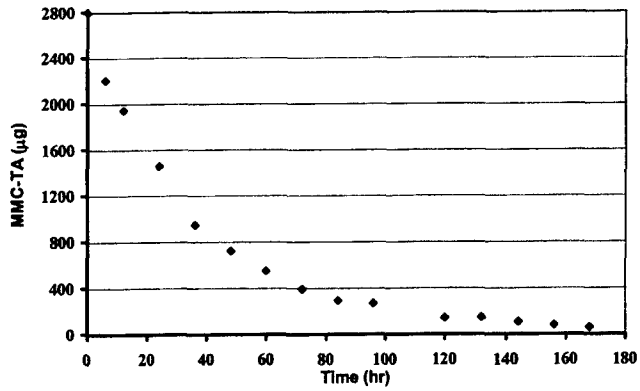

Figure 2. Hydrolysis of the MMC–TA conjugate in 0.1 mol/L phosphate buffer (pH 7.4) and propylene glycol (1:1) is graphically presented.

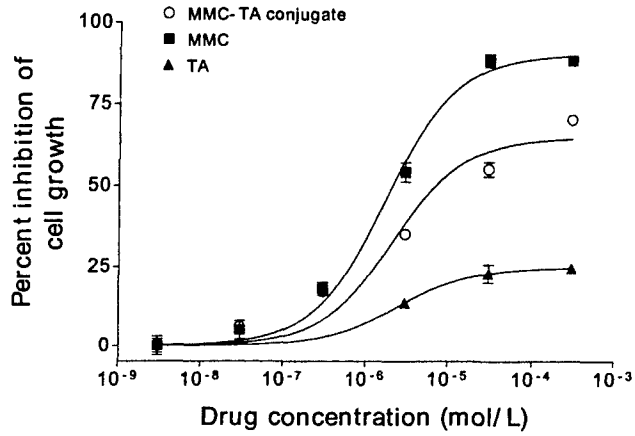

Figure 3. Dose response curve for MMC, TA, and the MMC–TA conjugate, showing different percentages of fibroblast cell growth inhibition (88, 25, and 67%, respectively) when compared to the DMSO similarly treated control.

Table 1. Antiproliferative Assay Results Showing the Maximal Percentage of Fibroblast Cell Growth Inhibition (Response Maximum) and the 50% Inhibitory Concentration (IC_{50}) of MMC, TA, MMC plus TA (1:1, Mixture), and the Conjugate MMC–TA

	IC_{50}[a] (μM)	response maximum (% inhibition)
MMC	1.7	88
MMC–TA conjugate	2.4	67
TA	2.5	25
MMC+TA mixture	1.6	83

[a] Concentration required to induce 50% of response maximum.

the evolution of TA and the TA–linker complex (9.73 and 5.71 min, respectively) confirmed the half-life of the conjugate (data not shown).

Antiproliferative Assays. Concentration–response curves for MMC, TA, and the conjugate-induced inhibition of NIH 3T3 cell growth are shown in Figure 3. The calculated response maximums for MMC and the conjugate were 88 and 67%, respectively. The IC_{50} (the concentration required to induce 50% of the response maximum) was calculated to be 1.7 and 2.4 μM for MMC and the conjugate, respectively (Table 1). The addition of unconjugated MMC and TA together resulted in an inhibitory response similar to that observed with MMC alone (Table 1). The addition of TA alone to the cells produced only limited inhibition of cell growth (25%) at the highest concentration tested.

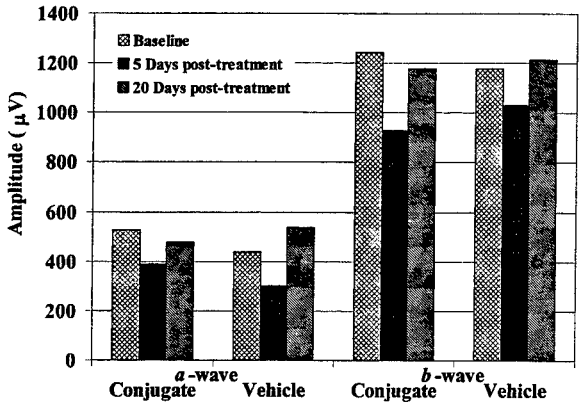

Figure 4. Average amplitudes of the electroretinogram *a*- and *b*-waves before (baseline) and 5 (3 rats) and 20 days (5 rats) postintravitreal injection of the MMC–TA conjugate (right eye) and the DMSO control (left eye).

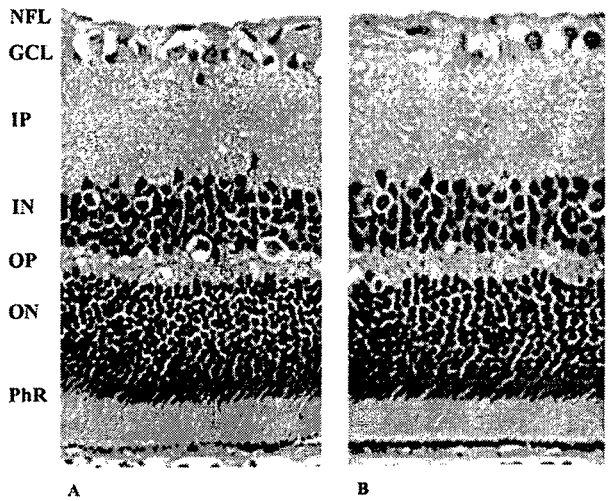

Figure 5. Histopathological sections of the neurosensory retina stained with hematoxylin and eosin (×40 magnification). (A) Left eye of control (R06) at day 5 postintravitreal injection of 2 μL, 99.9% DMSO; and (B) right eye of same rat at day 5 postintravitreal injection of 2 μL 0.39 mg/mL of MMC–TA conjugate. Legend: NFL, nerve fiber layer; GCL, ganglion cell layer; IP, inner plexiform layer; IN, inner nuclear cell layer; OP, outer plexiform layer; ON, outer nuclear cell layer; PhR, photoreceptor cell layer.

In Vivo Toxicity. Electroretinogram. Rats were injected intravitreally (2 μL) with 0.784 μg of conjugate or vehicle, and ERG *a*- and *b*-wave amplitudes were analyzed (Figure 4). Eyes that received intravitreal injections of the MMC–TA conjugate (right eyes) were compared to those that received dimethyl sulfoxide (DMSO) only (control, left eyes). There were no statistically significant differences between the amplitudes of the *a*- and *b*-waves at 5 or 20 days posttreatment. The slight reduction in ERG amplitudes observed at 5 days after treatment was attributed to the injection itself, as it affected both eyes equally.

Histopathology. The retina, by light microscopic examination, was normal in appearance at 5 and 20 days following the intravitreal injection of the MMC–TA conjugate, when compared to the DMSO control (Figure 5). No signs of retinal necrosis, photoreceptor cell loss, cystic degeneration, inflammatory cell infiltration, or hypocellularity of nuclear layers were observed microscopically in any rats.

Discussion

Our goal in this study was to link MMC (**1**) and TA (**2**) through a C_5 dicarboxylic acid moiety, in which the linker is connected by an ester bond with the primary alcohol function of TA and by an amide bond with the aziridine nitrogen of MMC. Both bonds were anticipated to hydrolyze in aqueous solution at physiological pH. Because the MMC–TA conjugate is a lipophilic molecule, it was expected to dissolve slowly, releasing both active ingredients of this codrug (MMC (**1**) and TA (**2**)) over a period of time. The C_5 linker was chosen because it is long enough to avoid sterical hindrance between the two principal building blocks. The glutaric acid linker was first connected to TA. To avoid a reaction of the secondary alcohol group in the 11-position with an excess of glutaric anhydride, only 1.1 equiv of it (relative to TA) was used. The final conversion of the TA linker to the MMC–TA conjugate (**5**) was easily accomplished by the addition of 3-fold excess of TA linker to MMC. The overall yield (51%) of MMC–TA (relative to MMC) was acceptable, and the procedure was not further optimized. The alternative strategy of attaching the glutaric linker first to MMC was less effective, producing low yields of the MMC–linker molecule (**3**) needed for the hydrolysis studies.

The conjugation of MMC and TA was confirmed by mass spectrometry and by 1D and 2D NMR spectroscopy. The positive electrospray ionization (ESI) mass spectrum revealed the molecular ion at *m/z* 887 $[MH + Na]^+$, which confirmed the molecular formula of $C_{44}H_{53}N_4O_{13}F$. The proton NMR spectrum showed all necessary signals, most of which were assigned with the help of the HSQC, HMBC, and H,H-correlation spectroscopy spectra. Also, the ^{13}C NMR spectrum showed all expected signals, which were assigned through the $^1J_{C-H}$ and $^nJ_{C-H}$ couplings observed in the HSQC and HMBC spectra. In the HMBC spectrum couplings between C-5′ of the linker and the protons attached to C-1″ and C-2″ of the mitomycin moiety on one hand and between 21-H_2 and C-1′ on the other hand clearly indicated that the linkage of the two compounds occurred in the desired fashion.

The conjugate of MMC and TA was hydrolyzed by cleavage of either the amide bond linking mitomycin and position 1′ of the glutaric moiety of the conjugate or the ester bond linking TA and position 1′ of the glutaric moiety of the MMC–TA conjugate (Figure 1). As a result, five compounds were identified in these studies: conjugate, TA linker, MMC linker, TA, and MMC. Analysis of the area under the curve (AUC)–time plots demonstrated that the evolution of TA–linker complex occurred faster than the evolution of TA indicating that the amide bond between mitomycin and position 5′ of the linker was more easily cleaved than the ester bond between TA and position 1′ of the linker. Normally, amide bonds are more stabile than ester bonds; however, this is not true for amide bonds whose nitrogen is part of an aziridine ring. Because of the strain on the aziridine ring, the free electron pair of the aziridine nitrogen cannot delocalize freely with the 5′-carbonyl and thus cannot contribute to a +M effect, which would normally decrease the reactivity of the amide C-5′ carbonyl.[18]

Once most of the conjugate had been hydrolyzed, the amount of TA linker decreased slightly due to cleavage of the ester bond between the TA and the linker. This is in agreement with the observed steady increase in the concentration of TA, even after most of the original TA–MMC conjugate had been hydrolyzed. The cleavage of this ester bond, however, appeared to be slower once the amide bond had been broken, since changes in the amounts of the compounds were only slight.

Similar quantitative observations could not be made for MMC and the mitomycin linker, since we were unable to completely separate their peaks by HPLC. The AUC of the combined peaks was fairly constant, once most of the conjugate had been hydrolyzed; thus, one can assume that mitomycin was reasonably stable under these conditions (aside from the conversion of mitomycin–linker complex to mitomycin). A control study supported this view, in which pure mitomycin was dissolved in phosphate buffer and propylene glycol to analyze the behavior of the compound under the same conditions as those used in the kinetic assay. Samples were taken every 24 h and analyzed by HPLC showing that no additional peaks (in addition to the mitomycin peak) appeared and the AUC of the single peak was fairly constant.

In the antiproliferative assay study, the MMC–TA conjugate and MMC showed a concentration-dependent antiproliferative activity at concentrations from 0.03 to 30 μM. The IC_{50} values for MMC and the MMC–TA conjugate were 1.7 and 2.4 μM, respectively. Although regression analysis demonstrated that the IC_{50} for MMC and the conjugate was not significantly different, the individual responses at concentrations above 3 μM, as well as the response maximum, were significantly less ($p < 0.05$) for the conjugate when compared to the parent MMC compound at the same concentration. We also compared the parent MMC response curve to the coadministration of equal molar concentrations of MMC and TA to determine if there were any interactions between the two drugs (MMC and TA) that contributed to the decreased efficacy of the conjugate. Although a slight difference between MMC (88%) in comparison to the MMC+TA mixture (83%) was observed, it could not alone explain the decreased efficacy of the conjugate. Hence, this reduction in conjugate activity at high concentrations is likely due to the conjugates limited activity before hydrolyzing and releasing the parent compounds, MMC and TA.

Intravitreal administration of the conjugate revealed no evidence of toxicity by ERG evaluation or histopathologic examination. The total ocular dose administered to these animals was approximately equivalent to the maximum effective concentration identified in the cell proliferation assay. These initial data support the idea that this MMC–TA conjugate will be relatively safe for various proliferative diseases including inflammatory and neoplastic disorders.

In summary, the newly synthesized MMC–TA conjugate (**5**) appeared to have similar hydrolysis kinetics as the previously described 5-FU–TA conjugate.[10,17] The conjugate also showed sufficient antiproliferative activity of the released compounds (MMC and TA) after its hydrolysis. The idea of using MMC in combination with triamcinolone was suggested after both agents were found to be effective in treating experimental proliferative ocular diseases,[19–21] thus taking advantage of the best characteristics of these two agents while minimizing their individual liabilities. The drugs become relatively insoluble when linked together. Hydrolysis of the conjugate allows the MMC and TA to be released slowly in equimolar concentrations. Ideally, a sustained drug delivery system should release the drug over a prolonged period of time, corresponding to the duration of disease activity. Our data suggest that the MMC–TA conjugate pharmacokinetics profile would be advantageous in the treatment of proliferative diseases affecting cavitary organs, where the short half-life of these agents has limited their clinical use.

Experimental Section

TA (2).[22] Triamcinolone, 800 mg (2.03 mmol), was suspended in 200 mL of acetone, and 20 drops of concentrated hydrochloric acid were added. The mixture was stirred under reflux for 1 h, during which triamcinolone was completely dissolved. The solution was stirred at room temperature for another 17 h. Thereafter, the mixture was poured into a solution of sodium bicarbonate (1 g/100 mL) and extracted with ethyl acetate. The combined organic layers were washed with a saturated sodium chloride solution, dried over potassium carbonate, and evaporated to dryness. Purification of the crude product was accomplished by column chromatography (silica, methylene chloride/methanol: 15/1) yielding TA (**2**), 458 mg (1.05 mmol; 52%), as a white solid; mp 275 °C (dec); R_f 0.42 (CH_2Cl_2/CH_3OH 15:1). ^{1}H NMR (400 MHz, acetone-d_6): δ 7.30 (d, J = 10, 1H, 1-H), 6.21 (dd, J = 10, J = 2, 1H, 2-H), 6.02 (dd, J = 2, J = 2, 1H, 4-H), 5.00 (dd, J = 3, J = 2, 1H, 16-H), 4.65 (dd, J = 9, J = 4, 1H, 21-H), 4.51 (s, 1H, exchangeable by D_2O, 11-OH), 4.40 (dddd, J = 11, J = 4, J = 3, J = 2, 1H, 11-H), 4.16 (dd, J = 9, J = 3, 1H, 21-H), 3.89 (br s, 1H, exchangeable by D_2O, 21-OH), 2.74 (dddd, J = 14, J = 14, J = 6, J = 2, 1H, 6-H), 2.60 (dddd, J = 29, J = 12, J = 12, J = 5, 1H, 8-H), 2.39 (ddd, J = 14, J = 5, J = 2, 1H, 6-H), 2.21 (ddd, J = 14, J = 4, J = 4, 1H, 12-H_e), 2.08 (m, 1H, 14-H), 1.93 (ddd, J = 14, J = 6, J = 6, 1H, 7-H_a), 1.73 (dd, J = 14, J = 2, 1H, 12-H_a), 1.63 (m, 1H, 15-H), 1.62 (s, 1H, 19-H), 1.50 (dddd, J = 14, J = 14, J = 14, J = 5, 1H, 7-H_e), 1.39 (s, 3H, acetonide-CH_3), 1.13 (s, 3H, acetonide-CH_3), 0.90 (s, 1H, 18-H). ^{13}C NMR (100.6 MHz, acetone-d_6): δ 210.8 (s, 20-H), 185.9 (s, C-3), 166.5 (s, C-5), 152.5 (d, C-1), 130.2 (d, C-2), 125.5 (d, C-4), 111.7 (s, $C(CH_3)_2$), 101.3 (d, C-9), 98.2 (d, C-17), 82.3 (d, C-16), 72.2 (dd, C-11), 67.4 (t, C-21), 48.9 (d, C-10), 45.9 (s, C-13), 44.1 (d, C-14), 37.4 (t, C-6), 34.1 (t, C-7), 33.9 (dd, C-8), 31.3 (t, C-12), 28.5 (t, C-15), 26.7 (q, acetonide-CH_3), 25.6 (q, acetonide-CH_3), 23.6 (q, C-19), 17.0 (q, C-18). UV (CH_3OH) λ_{max} (ϵ): 238 (18 400), 201 (10 300), 192 (7400). MS (pos.-APCI) m/z 435 M^+ (100).

TA-Glutaric Acid Linker (4).[22] TA, 260 mg (0.60 mmol), and glutaric anhydride, 78.0 mg (0.68 mmol, 1.13 equiv referred to TA), were placed into a 10 ml flask that had been previously flushed with argon. Then, 2.5 mL of dried pyridine was added, after which the mixture was stirred at room temperature for 18 h. The mixture was poured into 50 mL of ice water and acidified with 10% hydrochloric acid. After extraction with methylene chloride, the combined organic layers were evaporated to dryness, using toluene to remove the remaining pyridine. Purification of the crude product was accomplished by column chromatography (silica, methylene chloride/methanol: 15/1) to yield 268 mg (0.49 mmol, 83%) of TA linker (**4**) as a white solid; mp 220 °C (dec); R_f 0.23 (CH_2Cl_2/CH_3OH 15:1). ^{1}H NMR (400 MHz, acetone-d_6): δ 7.31 (d, J = 10, 1H, 1-H), 6.22 (dd, J = 10, J = 2, 1H, 2-H), 6.02 (s, 1H, 4-H), 5.15 (d, J = 18, 1H, 21-H), 4.94 (m, 1H, 16-H), 4.81 (d, J = 18, 1H, 21-H), 4.66 (br s, 1H, exchangeable by D_2O, 11-OH), 4.44 (m, 1H, 11-H), 2.75 (dddd, J = 14, J = 14, J = 8, J = 2, 1H, 6-H), 2.61 (dddd, J = 29, J = 12, J = 12, J = 5, 1H, 8-H), 2.42 (t, J = 7, 2H, 2′- or 4′-H_2), 2.52 (t, J = 7, 2H, 2′- or 4′-H_2), 2.39 (ddd, J = 14, J = 6, J = 2, 1H, 6-H), 2.25 (ddd, J

= 14, J = 3, J = 3, 1H, 12-H_e), 2.08 (m, 1H, 14-H), 1.93 (m, obscured by 3′-H, 1H, 7-H_a), 1.93 (t, J = 7, 2H, 3′-H_2), 1.84 (dd, J = 14, J = 2, 1H, 12-H_a), 1.68 (m, 1H, 15-H), 1.63 (s, 1H, 19-H), 1.50 (dddd, J = 13, J = 13, J = 13, J = 5, 1H, 7-H_e), 1.41 (s, 3H, acetonide-CH_3), 1.20 (s, 3H, acetonide-CH_3), 0.94 (s, 1H, 18-H). ^{13}C NMR (100.6 MHz, acetone-d_6): δ 204.3 (s, 20-H), 186.1 (s, C-3), 174.2 (s, C-1′), 173.0 (s, C-5′), 166.7 (s, C-5), 152.6 (d, C-1), 130.3 (d, C-2), 125.6 (d, C-4), 112.2 (s, $C(CH_3)_2$), 101.5 (d, C-9), 98.5 (d, C-17), 82.6 (d, C-16), 72.3 (dd, C-11), 68.1 (t, C-21), 49.0 (d, C-10), 46.4 (s, C-13), 44.2 (d, C-14), 37.4 (t, C-6), 34.4 (t, C-7), 34.1 (dd, C-8), 33.3 (t, C-2′), 33.1 (t, C-4′), 31.5 (t, C-12), 28.6 (t, C-15), 26.9 (q, acetonide-CH_3), 26.0 (q, acetonide-CH_3), 23.8 (q, C-19), 21.1 (t, C-3′), 16.9 (q, C-18). UV (CH_3OH) λ_{max} (ϵ): 238 (14 200), 200 (6800), 195 (6300), 191 (6000). MS (pos.-APCI) *m/z* 549 M^+ (100).

MMC–TA Conjugate (5).[17,22,23] A total of 216 mg (0.39 mmol) of TA linker and 65.0 mg (0.40 mmol, 1.02 equiv referred to TA linker) of carbonyldiimidazole (CDI) were dissolved in 3 mL of dried tetrahydrofuran (THF) (under argon), and the solution was stirred at room temperature for 4 h. Then, 37.0 mg (0.11 mmol, 0.29 equiv referred to TA linker) of MMC (Bristol Myers Squibb Oncology, Princeton, New Jersey) and 3 mg of (dimethylamino)pyridine (DMAP) were added, and the mixture was stirred at room temperature for 4 days. The reaction mixture was poured into 50 mL of water and extracted 3 times with chloroform. The combined organic layers were washed with water, dried over sodium sulfate, and evaporated to dryness. Purification of the crude product was accomplished by column chromatography (silica, chloroform/methanol: 10/1) to yield 59 mg (0.07 mmol, 43%) of the MMC–TA conjugate (**5**) as a purple solid. The purity of the compound was verified by two different HPLC gradient systems (system 1: R_{rel} 12.8 min, from $H_2O/CH_3CN/CH_3OH$ = 70%:24%:6% to $H_2O/CH_3CN/CH_3OH$ = 50%:40%:10% within 5 min; system 2: R_{rel} 3.4 min, $H_2O/CH_3CN/CH_3OH$ = 45%:45%:10% to $H_2O/CH_3CN/CH_3OH$ = 0%:83%:17% within 10 min; Waters Nova-Pak C_{18} 3.9 mm × 150 mm, flow rate for both systems: 1.5 mL/min). R_f 0.31 ($CHCl_3/CH_3OH$ 15:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.26 (d, J = 10, 1H, 1-H), 6.35 (dd, J = 10, J = 2, 1H, 2-H), 6.12 (br s, 1H, 4-H), 5.38 (br s, 2H, 7″-NH_2 or $CONH_2$), 5.09 (br s, 2H, 7″-NH_2 or $CONH_2$), 4.96 (d, J = 5, 1H, 16-H), 4.80–4.94 (m, complex, 3H, 10″-H and 21-H_2), 4.44 (m, obscured by 3″-H, 1H, 11-H), 4.44 (d, J = 13, 1H, 3″-H), 4.04 (dd, J = 11, J = 11, 1H, 10″-H), 3.67 (dd, J = 11, J = 5, 1H, 9″-H), 3.56 (dd, J = 13, J = 2, 1H, 3″-H), 3.51 (d, J = 5, 1H, 1″-H), 3.37 (dd, J = 5, J = 2, 1H, 2″-H), 3.20 (s, 3H, 9a″-OCH_3), 2.65 (m, 6-H), 2.48 (m, 2H, 2′-H_2), 2.46 (m, 2H, 4′-H_2), 2.40 (m, 1H, 6-H), 2.35 (m,1H, 12-H), 2.10 (m, 1H, 14-H), 1.95 (ddd, J = 7, J = 7, J = 7, 2H, 3′-H_2), 1.85 (m, obscured, 1H, 7-H), 1.78 (s, 3H, 6″-CH_3), 1.75 (m, 1H, 12-H), 1.65 (m, 1H, 8-H), 1.60 (m, 1H, 15-H), 1.60 (m, 1H, 7-H), 1.55 (s, 1H, 19-H), 1.42 (s, 3H, acetonide-CH_3), 1.19 (s, 3H, acetonide-CH_3), 0.94 (s, 1H, 18-H). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 204.0 (20-H), 186.2 (C-3), 183.0 (C-5′), 178.2 (C-5″), 175.2 (C-8″), 172.4 (C-1′), 166.0 (C-5), 156.6 ($CONH_2$), 154.0 (C-4a″), 152.0 (C-1), 147.2 (C-7″), 130.0 (C-2), 125.0 (C-4), 111.6 ($C(CH_3)_2$), 110.0 (C-8a″), 105.7 (C-9a″), 105.6 (C-6″), 100.1 (d, C-9), 97.8 (C-17), 82.0 (C-16), 72.1 (d, C-11), 68.0 (C-21), 62.0 (C-10″), 52.0 (C-9″), 50.0 (9a″-OCH_3), 49.0 (d, C-10), 49.0 (C-3″), 45.8 (C-13), 43.4 (C-14), 42.8 (C-1″), 39.8 (C-2″), 37.6 (C-12), 35.4 (C-4′), 34.1 (C-15), 33.8 (C-8), 33.5 (C-2′), 31.5 (C-6), 27.8 (C-7), 26.2 (acetonide-CH_3), 26.0 (acetonide-CH_3), 23.0 (C-19), 19.8 (C-3′), 16.3 (C-18), 8.0 (6″-CH_3). UV (CH_3OH) λ_{max} (ϵ): 356 (16 600), 234 (19 700), 212 (22 400). MS (pos.-ESI) *m/z* 887 (40) $[M - H + Na]^+$, H_R calcd for $C_{44}H_{53}N_4O_{13}FNa$, 887.3491; found, 887.3489, 833 (100) $[M - H - OCH_3]^+$.

Mitomycin–Glutaric Acid Linker (3). A 37.1 mg (0.11 mmol) amount of MMC was dissolved in 4 mL of dry THF under an argon atmosphere and treated with 14 mg (0.12 mmol, 1.1 equiv relative to MMC) of glutaric anhydride. After the solution was stirred for 72 h at room temperature, the solution was poured on ice, acidified with diluted hydrochloric acid, and extracted with chloroform. Purification was achieved by chromatography ((1) silica, CH_3OH. (2) Sephadex LH 20, CH_3OH) to yield 4 mg (8%) of MMC linker (**3**) as a purple solid. ^{1}H NMR (400 MHz, acetone-d_6): 6.39 (br s, exchangeable with CD_3OD, 2H, 7-NH_2 or $CONH_2$), 5.99 (br s, exchangeable with CD_3OD, 2H, 7-NH_2 or $CONH_2$), 4.95 (dd, J = 11, J = 4, 1H, 10-H), 4.43 (d, J = 14, 1H, 3-H), 3.98 (dd, J = 11, J = 11, 1H, 10-H), 3.65 (dd, J = 11, J = 4, 1H, 9-H), 3.59 (d, J = 5, 1H, 1-H), 3.48–3.53 (m, complex, 2H, 2-H and 3-H), 3.20 (s, 3H, 9a-OCH_3), 2.42–2.62 (m, 2H, 2′-H_2), 2.30 (t, J = 7, 2H, 4′-H_2), 1.82 (m, 2H, 3′-H_2), 1.80 (s, 3H, 6-CH_3).

Kinetics and Hydrolysis Studies. To determine its hydrolysis rate, 2.8 mg (3.3 × 10^{-6} mol) of the MMC–TA conjugate was dissolved in 2 mL of 0.1 M phosphate buffer (pH 7.4) and 2 mL of propylene glycol (PG).[24–26] The addition of PG was necessary to increase the solubility of the MMC–TA conjugate. The resulting solution was incubated at 37 °C. Samples of 25 μL each were taken after 6 and 12 h and each 12 h thereafter. The samples were immediately injected into the HPLC to study the hydrolysis of the conjugate. To determine absolute quantities of the hydrolysis fragments, four calibration curves were made available using pure MMC, MMC–TA conjugate, TA, TA linker, and MMC linker.

HPLC System. Column: M & W Chromatographietechnik Kromasil 100 C18, 250 mm × 4.6 mm, 5 μm; flow: 1 mL/min; mobile phase: methanol/water: 60/40 → methanol/water: 100/0 within 16 min, then methanol/water: 60/40 for 4 min; detection of the compounds at wavelengths of λ = 234 nm for MMC, TA, and TA linker; and λ = 356 nm for the MMC–TA conjugate.

The areas for the observed peaks were determined and plotted in an AUC–time plot. Because the two peaks for mitomycin and mitomycin linker could not be separated by HPLC, their AUCs were added and plotted in one plot. AUCs for the evolution of MMC were therefore not converted to absolute amounts (mg). Because of injection of the samples in a different solvent than was used for the calibration curves, the retention times changed somewhat but were verified by injecting pure samples of the compound in the same solvent. To study the behavior of pure mitomycin under the same conditions, mitomycin was dissolved in phosphate buffer and PG and also incubated at 37 °C. Samples were taken every 24 h and analyzed by HPLC.

Antiproliferative Assay. The fibroblast cell line (NIH 3T3) was cultured in Dulbecco's modified Eagles' medium containing 10% fetal bovine serum. Cells were plated at a density of 100 000 cells/well and allowed to grow for 24 h at 37 °C in 5% carbon dioxide in air.

The MMC–TA conjugate, MMC, and TA were dissolved in DMSO and diluted with media to achieve a final concentration between 3.0 × 10^{-4} and 3.0 × 10^2 μM. Each experiment was performed in triplicate. Cell proliferation was determined by means of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MMT) assay (Roche Molecular Biochemical; Mannheim, Germany). The assay was conducted according to the manufacturer's recommendations. MTT assay samples were measured using a spectrophotometer at an absorbance wavelength of 560 nm and a reference wavelength of 650 nm.

Toxicity to Rat Retina. Eight Wistar female rats, 4-weeks-old and weighing 70 g each, were used in this part of the study. Toxicity of the MMC–TA conjugate was assessed in response to intravitreal injections, using both functional (scotopic ERG) and anatomical (histopathology) analyses.

Our animal study was approved by the Institutional Animal Care and Use Committee. Research animal care and use at our institution meets all USDA and AAALAC approval requirements. Our study protocol adhered to the Association for Research in Vision & Ophthalmology Statement regarding the Use of Animals in Ophthalmic and Vision Research.

Intavitreal Injections. Two microliters containing 0.784 μg of MMC–TA conjugate dissolved in absolute DMSO was injected into the vitreous through the pars plana, under visualization with a microscope, following a paracentesis. This exposed the intraocular tissues to a concentration of approximately 30 μM of the MMC–TA conjugate, assuming a rat vitreous volume of 30 μL. The right eye of each rat received

the conjugate, while the fellow eye (left eye) received an intravitreal injection of 2 μL of DMSO and served as the control.

Electroretinogram. Animals were dark-adapted overnight and anesthetized with ketamine (11 mg/kg) and xylazine (14 mg/kg), their pupils were dilated with 1% atropine, and their corneas were anaesthetized with topical eye drops (0.5% proparacaine hydrochloride).

Setup. The optical system was adapted from Lyubarsky and Pugh[27] as described in Rohrer et al.[28] A clear plastic holder ending in a curved tip the shape of the cornea was used to provide full-field light stimulation to the rat's retina via a fiber optic light guide. The diffuser element, which had a recording electrode (0.2 mm platinum wire) glued to the inside, was gently placed against the cornea and electrical contact was completed through a drop of methylcellulose. Tungsten needle electrodes were placed in the neck and tail, to serve as reference and ground. Responses were amplified 2000-fold, band-pass filtered at 0.1−1000 Hz (2 pole Butterworth filter), and digitized with a 12 bit analogue to digital converter at 2 Hz. Data were stored, displayed, and analyzed with a PC interface and PClamp software. A single-channel optical bench was used for light stimulation.

Light Stimulus. The optical pathway consisted of a 250 Watt halogen lamp, and focusing lenses were used to focus the light beam to the end of the liquid light guide, a 500 nm band-pass filter, and a mechanical shutter. ERGs were recorded in response to 10 ms flashes at 40% maximum light intensities. Two responses were averaged, and the time between flashes was set to allow recovery of the *b*-wave between flashes.

Data Analysis. Flash ERGs were recorded prior to intraocular drug administration, to establish a baseline for *a*- and *b*-wave amplitudes, and 5 and 20 days after treatment. The *a*- and *b*-waves of the DMSO-injected eyes (left eyes) and drug-injected eyes (right eyes) were averaged (±standard error of the mean), and for statistical purposes, a standard *T*-test was employed, accepting a significance level of $p < 0.05$.

Histopathology. The eight female rats used for ERG analysis were euthanized (0.3 mL pentobarbitol) at 5 days (3 rats) and 20 days (5 rats) for histolopathological analyses. The animals were deeply anesthetized, and their eyes were enucleated and immediately immersed in a fixative consisting of 10% buffered formaldehyde solution and later processed in paraffin. Sections were stained with hematoxylin and eosin, and the coverslip was secured with DPX for light microscopy examination.

Acknowledgment. This work was supported by the Medical University of South Carolina, Institutional Research funds, 1999−2000; the South Carolina Commission of Higher Education; the U.S. Department of Defense; Research to Prevent Blindness; and NIH EY09741 (C.E.C.). The clinical expertise of D. Virgil Alfaro II, M.D., Farrow Counts, M.S., Todd Shearer, Ph.D., and Enrique-Roig, M.D., is gratefully acknowledged. The authors have no financial or proprietary interest in the products or equipment herein.

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JM010511B

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REPLY TO
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MCMR-RMI-S (70-1y) 28 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the technical reports identified on the enclosed list. Request the limited distribution statement for these documents be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

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